# IMPROVING PEANUT ALLERGY DIAGNOSIS USING SPECIFIC IMMUNOGLOBULIN E PEANUT COMPONENT AND BASOPHIL ACTIVATION TESTING

by

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# ABSTRACT

Peanut allergy is one of the most prevalent and deadliest food allergies. As public awareness has increased about the dangers of food allergies, there has been a corresponding increase in the research on peanut allergies. Significant increases in practical applications of our knowledge have occurred in the last few decades including questions that have been answered as to the best routes and methods of exposure to peanuts, there are innovative new therapies for possibly resolving peanut allergies, and cell based assays can now utilize components of peanuts that have significantly better predictive power and reproducibility than the previous whole peanut extracts. The next step is to be able to correlate laboratory tests to the severity of peanut allergy symptoms without having to expose the patients to the suspected food and risk urticaria, anaphylaxis, or even death. Basophil Activation Testing (BAT) is a promising way to do this and to help improve the correct diagnosis of potentially severe, life-threatening peanut allergy. The results from this study showed that BAT tests were able to correctly predict the outcome of food challenges as well as discriminate between peanut-sensitized and peanut allergic patients.

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# LIST OF ABBREVIATIONS

BAT: Basophil Activation Testing

DBPCFC: Double-Blind Placebo-Controlled Food Challenge

EDTA: Ethylenediaminetetraacetic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

FALCPA: Food Allergen Labeling and Consumer Protection Act

FDA: Food and Drug Administration

fMLP: formyl-Methionyl-Leucyl-Phenylalanine

HLA: Human Leukocyte Antigen

IgE: Immunoglobin E

IL3: Interleukin 3

IRB: Institutional Review Board

LEAP: Learning Early About Peanuts

MFI: Mean Fluorescence Intensity

OIT: Oral Immunotherapy

PCH: Primary Children's Hospital

PCI: Peanut Component Inconclusive

RBL: Rat Basophilic Leukemia Cell Lines

RAST: Radioallergosorbent Test

SIgE: Allergen-Specific IgE

SLIT: Sublingual Immunotherapy

SPT: Skin Prick Testing

SSC: Side Scatter Cells

WPI: Whole Peanut Inconclusive

# CHAPTER 1

# INTRODUCTION TO PEANUTS

## Peanut History

Peanuts originally came from South America and have been cultivated for thousands of years. Some of the earliest domesticated peanut seeds date back to 2000-3000 B.C. and peanut-styled ceramic pottery has been discovered in pre-Incan Indian cultures from circa 500-1000 A.D.[1] They have also been found entombed with ancient Peruvian mummies and appear to have been used in sacrificial offerings.[2]

Peanuts have spread around the world and can be found growing in almost any region. They were originally brought to North America by African slaves who used them to supplement their diets, and they were later grown and used as food for livestock in the 1700s.[1] Peanuts started gaining popularity in the 1800s when the Civil War broke out and food was scarce and then later when the Barnum Circus started selling roasted peanuts as they traveled around the United States.[2]

There was also an increase in peanut production due to George Carver Washington. He and other agricultural researchers encouraged Southern farmers to plant peanuts as a way to increase the amount of nitrogen in the soils which had been greatly depleted over the years by growing crops of cotton. He is credited with discovering at least 300 different uses of peanuts.

## Peanut Plants and Varieties

Peanuts are a legume that are grown underground in pods by a flowering plant in the pea family (Fabaceae). There are four basic types of commercial peanuts grown and eaten in the United States: Spanish, Virginia, Runner, and Valencia. Spanish peanuts have a high oil content and are more easily crushed. They are often turned into peanut butter. Virginia peanuts have a large kernel and are often sold unshelled or as shelled salted peanuts. Runners are technically a hybrid between Spanish and Virginia peanuts, but they have been used for many years and are often considered as a separate variety. Valencia are sweeter and are most often used for boiled peanuts. Studies have shown that all the varieties are similar in terms of total protein, and in the content of the two most allergenic proteins Ara h 1 and Ara h 2.[3] Refer to Table 1.1 for a compilation of the exact percentage of proteins and attributes of the top four varieties.

Table 1.1 Peanut Attributes of the Top Four Varieties in the United States

| | **Spanish** | **Virginia** | **Runner** | **Valencia** |
|---|---|---|---|---|
| Mainly used for | Peanut Butter, Candies | Eaten In-shell, Salted Peanut | Peanut Butter | Boiled Peanuts, Roasted/Unshelled |
| Attributes | High oil content, "nutty" flavor | Large kernel | High yields, medium size | Very sweet |
| Total Protein | 27-29% | 26-29% | 24-28% | 26-29% |
| Ara h 1 protein | 12-16% | 15-16% | 13-16% | 14% |
| Ara h 2 protein | 5.9-9.2% | 6.6-8.3% | 6.2-7.7% | 8-9.3% |

In general, peanut total protein has been found to make up 24-29% of the whole peanut with Ara h 1 accounting for 12-16% and Ara h 2 for 5.9-9.3% of the protein fraction.

It is important to note that different countries do have different peanut breeding strains and it has been shown that the allergen content can vary somewhat; for example, one peanut line from India has a 7% Ara h 1 content whereas Nigeria has a peanut strain that has 18.5% Ara h 1.[4] The different varieties of peanuts also have different expression patterns of the main allergens during seed development but the allergens are restricted to seed and are not found in the leaves, flowers, or roots of the peanut plant.[5]

The method of preparation of peanuts has been shown to change the allergenicity. Roasting peanuts has been shown to increase allergenicity and can bind antipeanut antibodies at a 90-fold increase versus raw peanuts.[6] In contrast, boiling or frying peanuts has been shown to decrease the allergenicity and has been suggested as a possible reason why peanut allergens are less prevalent in China and parts of the world where peanuts are commonly eaten boiled rather than roasted.[7]

## Peanut Components

Historically, skin prick testing and serum IgE antibody testing has been done using the whole peanut extract, but this has been problematic over the years due to different manufacturers using different varieties of peanuts and different preparation methods (eg, raw versus roasted peanuts). A newer method that has been gaining interest over the years is to break down the whole peanut into its different protein components. These individual proteins are present in whole peanut extracts and have been shown in

the past to differ between different manufacturers[8] and can even possibly differ between lots. Certain components are also only found in trace amounts in the whole peanut extract. By creating a test specifically for the peanut component instead of the whole extract, there can be greater standardization of test results.

Since the amino acid sequences and protein structures of the peanut components are known, they can be replicated by using Escherichia coli (E. coli) strains or phages that have been given cDNA for the specific component. This further eliminates variability and the risk of contamination of other peanut components that might be present if an extraction process using whole peanut is used.

These components have been labeled sequentially as Ara h 1, Ara h2, Ara h 3, and so forth. The "Ara h" is derived from the scientific name for peanut, *Arachis hypogaea*. Table 1.2 lists the components that have been discovered and registered with the World Health Organization and International Union of Immunological Societies. Ara h 4 is somewhat unique in that it was originally given its own designation but was later discovered to be an isoform of Ara h 3 and renamed Ara h 3.02. Some components have been proven to be more important than others in eliciting the severity of peanut allergy. The most commonly studied and addressed components are Ara h1, Ara h2, Ara h3, Ara h6, Ara h8, and Ara h9.

Ara h1 has been identified as a seed storage glycoprotein (7S vicilin-like globulin). It forms a stable trimer and has several hydrophobic residues that are critical for IgE binding.[9] While there are not differences in Ara h 1 levels between the main U.S. cultivars, there has been a difference described between the size and maturity of the peanut. Jumbo and medium-sized peanuts were shown to have higher levels of Ara h 1

Table 1.2 Identified and Registered Peanut Components

| Component Name | Protein/Biochemical Name | Biological Function |
|---|---|---|
| Ara h 1 | 7S vicilin-like globulin | Seed storage glycoprotein |
| Ara h 2 | 2S albumin/conglutin | Trypsin inhibitor |
| Ara h 3 | 11S globulin/glycinin | Trypsin inhibitor |
| Ara h 4 | 11S globulin/glycinin (isoform of Ara h 3) | |
| Ara h 5 | Profilin | Cell motility/actin dynamics |
| Ara h 6 | 2S albumin/conglutin | |
| Ara h 7 | 2S albumin/conglutin | |
| Ara h 8 | PR-10 protein (Bet v 1-homolog) | Plant defense protein |
| Ara h 9 | Nonspecific lipid transfer protein | Lipid transfer protein |
| Ara h 10 | 16 kDa oleosin | Lipid Storage |
| Ara h 11 | 14 kDa oleosin | Lipid Storage |
| Ara h 12 | Defensin | Host defense peptides |
| Ara h 13 | Defensin | Host defense peptides |
| Ara h 14 | 17.5 kDa oleosin | Lipid Storage |
| Ara h 15 | 17 kDa oleosin | Lipid Storage |
| Ara h 16 | Nonspecific lipid transfer protein 2 | Lipid transfer protein |
| Ara h 17 | Nonspecific lipid transfer protein 1 | Lipid transfer protein |

than smaller more immature peanuts.[10]

As was previously mentioned, roasting increases the antigenicity of peanuts. Ara h 1 in particular increases by 22-fold after roasting.[10] It's also been found that Ara h 1 and Ara h 2 are more resistant to heat and gastric digestion after undergoing a Maillard reaction, a common nonenzymatic reaction that occurs in cooking/roasting that causes 'browning' and gives food more flavor.[6] In contrast to roasted peanuts, boiled and fried peanuts have been shown to have no detectable amounts of the Ara h 1 trimer form and markedly reduced amount of the Ara h 1 monomeric form.[7]

Importantly, Ara h 1 peptides can change conformation at different pHs and it has been proposed that peptide fragments that survive the stomach's low pH environment and

make it to the small intestine's higher pH environment can undergo conformational change and form large aggregates with newly uncovered allergen epitopes[11] which might cause delayed allergic responses. Once peanut protein reaches the intestinal epithelia, the way it gets absorbed is unique. In an experiment using Caco-2 cells (a human intestinal epithelial cell line), Ara h 1 and h 2 have been shown to disrupt the transmembrane tight junction proteins[12] and, in essence, escape from the GI tract. These results have been suggested as an explanation for the increased allergenicity of peanuts.

Ara h 2 is a 2S albumin/conglutin protein that is known to function as a trypsin inhibitor. It is considered the most predictive and accurate indicator of peanut allergy when compared to the other peanut components and whole peanut extract. *Nicoaou et al* showed that it possessed significantly increased sensitivity and specificity compared to the whole peanut extract and the other peanut components. At levels of 0.35 kU/l, whole peanut had a sensitivity of 96.55% and specificity of 26.92%, but Ara h 2 had a sensitivity of 100%, and specificity of 96.08 %[13] and has also been shown to be a more potent allergen then Ara h 1 in functional IgE-crosslinking assays.[14] It has also been shown that heating of Ara h 2 may enhance its binding to intestinal epithelium cells, performed with the Caco-2 cell line, and that in mouse models, heated Ara h 2 induces significantly higher levels of IL-2 and IL-6 from splenocytes then unheated Ara h 2.[15] Comparison of fried and boiled peanuts versus roasted peanuts showed that unlike the Ara h 1, Ara h 2 had the same amount of protein and antigen in both preparations. IgE-binding to both Ara h 2 and h 3, however, was reduced in the fried and boiled peanuts[7] and roasting of Ara h 2 increased its trypsin inhibitory activity by 3.6 fold.[16]

There have also been studies into the cross-reactivity with the different

component antibodies indicating that IgE binding to Ara h 2 can be inhibited by 49-89% by adding Ara h 1 and Ara h3.[17] Ara h 3 is an 11S globulin/glycinin protein that also functions as a trypsin inhibitor. It also includes Ara h 4 which was originally given its own designation but was later found to be an isoform of Ara h3 and is now considered to be one antigen. Together, Ara h 1, h 2, and h 3 represent the most common peanut antigens found to cause significant peanut allergies. IgE antibodies directed against these proteins are more indicative of severe peanut allergy and a greater risk of anaphylactic shock.[18]

Ara h 5 is a profilin that helps with cell motility and actin dynamics. Tests for antibodies against Ara h 5 are not currently commercially available since it is considered a more minor peanut allergen. It has been reported that only 13% of patients allergic to peanuts are sensitized to Ara h 5.[19]

Ara h 6 is a 2S albumin/conglutin that has a 59% amino acid sequence match to Ara h2. Ara h 6 has been shown to be epitopically similar to Ara h 2 and that either one can restore allergenicity in whole peanut extract; consequentially, they have been labeled as "substantially redundant."[20] Ara h 6 has also been shown to have similar properties in comparison to Ara h 2 in that it is resistant to pepsin digestion and heat treatment.[21] Sensitization to Ara h 6 has been shown to be strongly correlated with Ara h 2 sensitization ($r = 0.861$).[22] Indeed some studies have reported that all patients with Ara h 2 specificity showed matching Ara h 6 specificity,[23] but larger studies have noted small numbers of patients 3/166 (2%) that have sensitization to only Ara h 6.[24]

Ara h 7 is also a 2S albumin/conglutin like Ara h 2 and Ara h 6; however, it only has a 35% amino acid match to Ara h 2.[35] Although it has been reported to have a

moderately high sensitization rate of 43% in two different studies,[24,19] it is not commercially available for routine laboratory testing.

Ara h 8 is a Bet v 1 homolog panallergen. Bet v 1 is one of the major antigenic proteins expressed by Birch trees (*Betula verrucosa*) and is considered a panallergen because of its similarity to other antigens expressed by fruits and other trees, especially those in the Fagales genus. One study, performed by *Moverare et al*, found that all of their patients who had Ara h 8 antibodies also had antibodies against birch pollen and they were highly correlated ($p<0.0001$).[18] Bet v 1 has also been shown to inhibit SIgE binding to Ara h 8 in immoblotting and RAST inhibition assays.[25] Patients that become sensitized to Bet v 1 and its homologs most commonly have cross-reactivity with allergens in peach, apple, celery, and carrot which are correlated with oral allergy syndrome.[26] Ara h 8 has been studied and found to be indicative of mild to negligible peanut allergies when antibodies against Ara h 1, 2, and 3 are not present.[27] It has also been shown that Ara h 8 antigenicity is actually decreased by 9-fold during roasting, as determined by inhibited IgE binding, and that the enzyme pepsin, found in the stomach, can completely destroy the antigenicity within 5 seconds.[25]

Ara h 9 is a nonspecific lipid transfer protein that has some similarity to peach antigens (62-68% amino acid overlap).[28] Ara h 9 is an interesting allergen in that it is considered significant in certain populations, specifically Mediterranean populations,[28,29] but is considered a more minor allergen in almost all other populations. A recent study, however, has shown that Ara h 9 sensitization is linked to a higher prevalence of bronchospasms (26% vs 9%) and may be more significant than previously thought.[30]

The allergens Ara h 10 thru Ara h 17 have not been extensively studied at this

point. These allergens have been identified as either oleosin's, defensin's, or lipid transfer proteins.[31]

## Peanut Allergies and Sensitization Routes

It was uncommon before the late 1800s for allergies to occur and studies done around 1870 showed "hay fever" as an emerging disease.[32] Peanut allergies are reported to have started showing up in the early 1900s although most of the medical community's focus was still on the more common food allergies like milk and eggs at that time.[1] Peanut allergies are currently a significant problem that affects approximately 1.2% of children, and over three million Americans are affected by peanut and/or tree nut allergies.[33] Peanuts and tree nuts have been shown to account for more than 90% of all fatal anaphylactic reactions and approximately 100-200 people die every year in the United States from anaphylaxis due to accidental peanut ingestion.[34,35]

There is a significant portion of the atopic population that is extremely allergic to peanuts and can have life-threatening reactions from very small amounts of peanut protein. Peanut allergy has become a real fear for many parents as they try to keep their kids from developing it or from having life-threatening reactions if they do develop an allergy. It is common to walk into schools, daycare centers, or cafeterias and see signs which ban peanuts or discourage peanuts from being brought into the area. One third of individuals allergic to peanuts also have allergies to tree nuts[36] which is why tree nuts are usually included in the 'bans' as well. One recent study tried to investigate the spread and distribution of peanut protein in homes and in the school environment. Their results showed that peanut proteins can still be present in saliva and on a person's hands for 3

hours after consumption and that the proteins can be aerosolized when the peanuts are being shelled.[37] The risk of systemic reaction from skin contact to peanut butter has also been shown to be rather low in children with significant peanut allergies,[38] indicating that the 'ban' on peanuts may be unnecessary in preventing life-threatening reactions. Reactions such as redness, itching, and wheals, however, are still seen in children with significant peanut allergies upon contact with peanut butter which might be avoided with peanut bans.

The best way to stop an allergy is to prevent it from occurring. For a long time, it was debated as to whether or not children should be exposed to peanuts from an early age. It was thought that if you prevent exposure, you could prevent the allergy. Starting in the 1990s, medical professionals such as the American Academy of Pediatrics started recommending avoidance diets for young children. Pregnant or nursing mothers were also advised to avoid peanuts as well to limit the children's exposure through secondary sources. These avoidance diets for mothers and children did not slow the increase in peanut allergies and questionnaire studies done by *Sicherer et al* showed that peanut allergy in children actually increased from 0.4% in 1997 to 0.8% in 2002 and then to 1.4% in 2008.[39]

Several studies started to look at the routes of peanut sensitization and had suggestive results that early oral exposure had a protective effect.[40] Most of these studies were questionnaire based, however, or included only a small population size. To come up with a definitive answer, the Learning Early About Peanuts (LEAP) study was established. This study enrolled 640 at-risk children who already had an egg allergy or severe eczema, and either put the children on a peanut containing regimen or a peanut-

avoidance diet and followed them for 5 years. The recently published LEAP results indicated a significant and definitive decrease in peanut allergies (13.7 % vs. 1.9%) in children that ate peanuts on a regular basis.[41] Early exposure through the skin by application of peanut-oil containing creams; however, instead of by dietary exposure, has been linked to an increase in peanut allergies.[42]

Older studies reported no effect of maternal peanut consumption on their children's development of peanut sensitization.[40,42] There have also been recent studies, however, suggesting that consumption of peanuts and tree nuts by nonallergic mothers during pregnancy and while nursing lowered the risk of peanut and tree nut allergies in their children.[43]

Other environmental factors like Vitamin D levels, family size, season and method of birth, geographical location, and the presence of pets in a household have also been cited as possible factors in the development of asthma and allergies. Lower vitamin D levels have been shown to be associated with allergic disease and elevated serum IgE[44] as well as increased severity of atopic dermatitis in children with allergies.[45] It has also been reported that mothers who took vitamin D during pregnancy had children with a reduced risk of wheeze and eczema.[46] Researchers found that acute allergic reactions are more common in the northeastern part of the U.S. than in the Southern U.S., which has been hypothesized as being related to sun-exposure and vitamin D levels.[47] Seasonal factors have also been investigated since many factors change over the course of a year—time of sunlight per day, pollen levels, time spent indoors and exposure to indoor allergens. Children born in autumn and winter have been shown to have a higher prevalence of allergic rhinoconjuctivitis and are more likely to have sensitization to

common foods like egg white, milk, and wheat.[48] It has also been reported that children born by cesarean section are at an increased risk of asthma and atopy, which may be related to disruptions or delayed colonization of the infant's gut with beneficial microbiota.[49]

Exposure to more outdoor allergens may be important in preventing allergies. Children living in rural areas have a lower prevalence of food allergy than those living in urban centers (6.2% vs 9.8%).[50] Children in rural areas also have other lifestyle differences such as greater number of siblings, less exposure to tobacco smoke, and are more likely to have pets or be exposed to animals, that could account for the decreased prevalence of atopy.[51] A greater number of siblings and exposure to pets during the first year of life is associated with a lower prevalence of allergic rhinitis and asthma.[52] It has also been proposed that children's exposure to air pollutants, such as diesel particles, in urban centers can contribute to the development or worsening of allergies and asthma by inducing inflammatory responses.[53,54]

Sensitization may also be related to blood transfusion, bone marrow, and organ transplants. Blood transfusions can result in passive sensitization. It has been shown that donor IgE antibodies can sensitize recipient's mast cells and basophils within 3 hours after transfusion and that, in one blood collection center, 23% of blood donors had significant levels of IgE antibodies to common allergens.[55] Peanut allergens have been shown to be passively transferred from a donor's blood and have caused anaphylaxis in at least 1 recipient. There has also been one instance where a patient developed a serious anaphylaxis nut allergy after receiving a liver transplant from a boy that had died from anaphylaxis due to accidental peanut ingestion.[56] The liver may be more prone to

transferring allergies then other organ transplants due to the pluripotential hematopoietic stem cells and dendritic cells that reside in the liver.[57] Food allergy transfers have also been reported in heart, lung, kidney, and intestinal transplants, and the allergy is more likely to transfer if the recipient is a child.[58] Most of the transferred food allergies have been reported to resolve after a few years.[58]

## Peanut Exposure

Exposure to allergens can be deadly for those children that do have severe food allergies. Even small amounts of contamination have been shown to have serious effects.[59] The Food Allergen Labeling and Consumer Protection Act (FALCPA) was implemented in 2004 in response to the growing danger of allergen contamination and exposure. The act requires food manufacturers to indicate if a product has a major food allergen. The act defines eight major food allergens: milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans. These eight food allergens are said to account for over 90% of all documented food allergies in the United States.[60] Food manufacturers have made great strides in labeling foods since the act went into effect in 2006; however, the FDA has not established any threshold levels for any of the allergens. This has led to inconsistent labelling between companies. Some companies put generalized contamination statements on food products such as "this product was produced in a facility that also process peanuts" even though the risk is extremely low, or they simply put every one of the eight major allergens so they have no liability concerns, whereas other companies with much higher chances of contamination will not add such disclaimers. The language of the disclaimer is also not standardized and may vary from

"shared facility," "shared equipment," and "may contain." This can be frustrating and confusing to those that have food allergies.

The question of how much contamination is in the food is a real concern. The issue is further complicated by the fact that every allergic patient will have a different level at which they will react to the food antigen. A study in 2007 tested 200 different food products with disclaimers and only detected clinically significant contamination in 13 products.[61] Another study found that peanut was only found in advisory label products 7.3% of the time but 33% of the time when it was labeled as a minor ingredient.[62] Research has shown that consumers are increasingly ignoring food contamination disclaimers and from 2003 to 2006 the percentage of those heeding the warnings dropped by ten percent.[61]

*Ballmer-Weber et al* recently did a study where they looked at what concentration level a food had to get to before it provoked a response in allergic patients. They then defined a threshold in which, theoretically, only 10% of allergic patients would have a reaction. Peanut's contamination cut-off level was defined as 0.03 mg.[63] Their study lacked patients that had a history of anaphylaxis so it does not display a true reflection of the general population. It does, however, provide a good starting point under which manufacturers should try to get their levels of contamination.

Some have suggested that manufacturers could also test for contamination in the factories and put the level of contamination on their products (ie, "This product was produced in a facility that also processes peanuts. Contamination levels may reach up to 0.01 mg"). Thus, every person would be able to evaluate whether or not they could safely consume the product. This is problematic for several reasons. The first is that

different lab tests might use slightly different epitopes or kits and the results may not be standardized. The second reason is that the antigenic epitopes can be altered by different cooking methods (ie, heating, baking). It is also difficult to get consistent results with allergens when they are in different food matrixes. It has been shown that fat content can alter the levels of peanut detected in ELISA-based assays and that allergic patients are able to ingest more peanut protein when they are in high fat recipes.[64]

## Methods of Diagnosis of Food Allergies

### *Double-Blind Placebo-Controlled Food Challenge (DBPCFC)*

The DBPCFC has been considered the gold standard in allergen testing for many decades. The principle behind it is relatively simple. The food suspected of causing a reaction is given to a patient in increasing doses to see if it does, in fact, cause a reaction. This inherently makes the DBPCFC a dangerous and unappealing test to patients.

There are several problems that have to be addressed when doing food challenges. The first is that stress and anxiety can cause false positives so placebo doses must be mixed in with the true doses to make sure results are accurate. The second is that anti-histamines, other anti-allergy drugs, or immunosuppressive medications can interfere with the results and must be discontinued several days or weeks before the challenge. The third and most important problem is that when a reaction is provoked, it can quickly become life-threatening and medical personnel must be present at all times and ready to treat any reaction that occurs.

The problems that have to be addressed when performing DBPCFCs make them costly and time-intensive but they do provide a clear picture of exactly what effect a

specific food will have on a patient.

*Skin-Prick Testing (SPT)*

SPT techniques have been utilized since 1865.[65] The technique is relatively simple, in which a small amount of allergen is placed in the skin through pricking or scratching the skin. Histamine and saline solutions are used as controls. Any wheals that develop after 15 minutes are indicative of sensitization and the larger the wheal the more likely that the patient has a true allergy. Three millimeters wheals and larger are generally considered positive for an allergy. However, there are some differences between manufacturers and between single and multiheaded skin testing devices and it has been recommended that three millimeters is not arbitrarily used as a positive threshold.[66] A skin prick result of greater than 8 millimeters has been shown to have a high predictive value for clinical allergy to peanut.[67]

Multiple factors can affect SPT such as the location on the body, sun damage of the skin, allergen extract quality, and certain medicines such as antihistamines, antidepressants, and steriods.[68] The repeatability of skin prick testing has been questioned and investigated by several researchers. One study, done over the course of 3 years, found that a positive SPT had a repeatability of 100% when supported by a history of allergy; however, it drops to 67% when there was no history of allergy.[69] A twenty-year study found that skin prick test positivity done at 5 years of age had a reproducibility of 100% at 6 and 15 years later and did predict allergy symptoms; however, many new positive allergens did occur over that time span so its sensitivity was not high (23-28%).[70] The scoring and interpretation by doctors is also something that must be taken into

account. It has been shown that there can be significant interphysician variation on results that are not strongly positive or negative.[71]

Currently whole peanut extract derived from native peanut is most commonly used in allergy clinics. It is also possible to do SPT testing with recombinant peanut components and it has been suggested that this may yield more consistent results.[72] Studies trying to correlate peanut component SPT with the severity of the peanut allergy have been performed. It was found that when researchers did the SPTs at multiple concentrations the more severe patients had significantly higher SPT reactivity to Ara h 2 and Ara h 6 at low concentrations,[73] indicating that perhaps when diagnosing peanut allergy two or three different concentrations of allergens could be used to further evaluate the severity of the allergy.

*Blood Tests –RASTs & ImmunoCAP*

The first blood tests were radioallergosorbent tests (RASTs). These tests detected a patient's IgE antibody level to a specific food by using purified food antigens and anti-IgE antibodies that were bound to a radioactive isotope. Later blood tests used the same principle but had an enzyme bound to the anti-IgE antibodies that create either a fluorescent or luminescent signal instead of a radioactive one.

Blood tests have several advantages and disadvantages. Their major advantage is that they are very convenient and easy for the patient. There is also no risk of anaphylactic reactions and the test is unaffected by anti-allergy drugs so patients do not have to stop taking their medications. One of the disadvantages is that antibodies can cross-react and cause false positive reactions. The second disadvantage is that it can only

indicate sensitization to allergen and not whether the patient would actually react to the allergen. Studies looking at the utility of IgE tests have, therefore, had to set somewhat high concentrations of antibodies to achieve good positive predictive values. These cut-off levels for 95% positive predictive value vary from 15 kU/L to 24.1 kU/L.[67,74] It is also important to note that it is recommended that tests done with different methodologies not be compared even if they have the same units of measurements.[68]

*Basophil Activation Tests (BAT)*

BAT testing is a new type of testing that has not yet achieved widespread use in the allergy community. BAT-allergy testing is based on the principle that if a person is allergic to something, they have specific IgE antibodies that circulate in the blood and that also bind to basophils and mast cells. Then, when the bound antibodies encounter the antigen for which they are specific, they set off a cascade that leads to basophil degranulation. Degranulation in turn leads to the expression of CD63 on the cell surface which can be quantified by flow cytometry. BAT testing is further discussed in Chapter 4.

## Treatments for Peanut Allergy

The percentage of children that outgrow their peanut allergy tends to be lower than most other common food groups. A small study done in 1989 originally reported no natural resolution in atopic individuals but they had a small population size ($n$=36).[75] More recent studies done with large populations have shown that approximately 21.5% do outgrow their peanut allergy.[76] Currently, for children that do not outgrow their

allergy, there are several different treatment options available: avoidance diets, oral immunotherapy, subcutaneous immunotherapy, probiotic oral immunotherapy, and anti-IgE therapy.

Avoidance diets are actually the absence of treatment. In these cases, the patient is usually told to avoid eating peanuts and all possible exposures to peanuts. If the allergy is severe, they may also be given epinephrine shots (EpiPen's) in case they do accidentally ingest peanuts and go into anaphylactic shock. Avoidance diets do prevent adverse reactions, but they do not resolve the allergy.

Sublingual immunotherapy (SLIT) and oral immunotherapy (OIT) are two approaches to desensitizing patients to allergens. In SLIT, small amounts of allergen extract are placed under the tongue daily. In OIT, the small amounts of allergens are swallowed and ingested daily. In one double-blind, placebo-controlled study, they found that children undergoing SLIT were able to safely ingest 20 times more peanut protein then the placebo group after 1 year of treatment.[77] OIT studies have had similar results.[78] There are dangers to using SLIT and OIT, however, including the risk of anaphylaxis and life-threatening allergic reactions. Most participants also report minor allergic reactions throughout the treatment process.[79]

The long-term results of SLIT and OIT also need to be further evaluated. One study found that 4 weeks after finishing peanut OIT only 50% of the patients were able to pass a food challenge.[80] Another disadvantage of SLIT/OIT is that it does take months to years to complete the therapy and adherence to the daily doses can be poor. One study found that over 50% of patients on a 3-year peanut SLIT discontinued therapy before completing a full course.[81]

Using probiotics to treat allergies is a recent innovative idea. Immunological studies have shown that probiotic bacteria by-products like short chain fatty acids can increase extrathymic generation of T regulatory cells.[82] One recent study done in Australia enrolled 62 children to undergo oral immunotherapy and then they gave half of the children the probiotics bacterium *Lactobacillus rhamnosus* to take alongside their scheduled peanut protein doses. The other half of the enrolled children functioned as a placebo control group and they received no probiotics and placebo doses consisting of maltodextrin with brown food coloring and peanut essence. They found that 82.1% of the probiotics groups achieved sustained unresponsiveness after the treatments, whereas only 3.6 % of the control group achieved sustained unresponsiveness.[83] Even more surprising was that food challenges performed 2 to 5 weeks later after patients were put back on a peanut elimination diet showed that 23 out of 25 patients were still tolerant to peanut. The researchers are planning on doing further food challenges on a later date to determine how long lasting the tolerance is.

It is important to note that while the probiotic immunotherapy research is promising, there are also dangers to using probiotics. Four major probiotic metabolites (butyric acid, flagellin, lipoteichoic acid, propionic acid) that are currently being studied and have been shown to have health benefits have also been linked to a wide range of disease and conditions such as cancer, mental retardation, synergistic enhancement of endotoxins/inflammation, and long-term behavioral deficits.[84] More research into mechanisms of how probiotics work and which strains are the safest still needs to be performed. It has also been suggested that certain patients such as neonates or those with immunodeficiency should use probiotics with caution as reports of sepsis linked to

probiotic use have been reported.[85]

Another important treatment option is Anti-IgE therapy with Xolair, also known as omalizumab. It has been shown that the frequency of peanut-specific T cells is higher in those with peanut allergy and that IgE depletion decreases the proliferation of those cells.[86] Xolair is an antibody that binds to a CH3 domain on IgE molecules and effectively neutralizes free IgE in the serum and is considered an effective therapy for food allergies, asthma, atopic dermatitis, and chronic urticaria.[87] It has been shown to downregulate FcεRI on mast cells and basophils to <5% within a few months of the start of treatment.[88] Xolair is FDA-approved for adults and children starting at age 12. It is injected under the skin every 2 to 4 weeks by a healthcare provider. There are also some major risks to taking Xolair, including anaphylaxis, fever, muscle aches, rash, parasitic infections, and the more common side effects seen include pain in arms/legs, dizziness, nausea, headaches, cough, joint pain, and upper respiratory tract infections.[89] The effectiveness of anti-IgE treatment can be monitored by measuring the amount of allergen needed to cause basophil activation, called CD-sens, which has been shown to correlate well with determining a patient sensitivity level as they undergo anti-IgE treatment.[90] CD-sens will be discussed further in Chapter 4.

# CHAPTER 2

# EXPERIMENTAL SETUP

## Recruitment of Patients

This research study was reviewed and approved by the University of Utah Institutional Review Board (IRB #84399: Basophil Activation in Allergic Disease.) Recruitment of patients was done at Primary Children's Hospital (PCH) in the pediatric allergy clinic over the course of several months. All patients gave written informed consent or assent along with parental informed consent before entering the study.

A total of 47 peanut-allergic patients were recruited from PCH. All PCH patients had either positive SPT or detectable SIgE levels to peanut and all patients had varying levels of clinical allergy symptoms. These patients all had SIgE levels and basophil activation testing performed on their blood. A total of 7 atopic patients being seen for other allergies and conditions were also recruited from PCH during the same time period and were all clinically tolerant to peanut. The median age for the two groups were comparable (6.5 years vs 8.3 years)($p$=0.497). The percentage of males/females for the PCH group was pretty evenly split with females accounting for 55% of the study population. The control group had a lower percentage of female participants (0.29%) which has due, in part, to the group's much smaller sample size and the randomness of sampling. As peanut allergies are not known to significantly differ between males and

females, this difference is not clinically relevant.

The patients were put into five different groups based on their SPT results and clinical presentation. Refer to Figure 2.1 for a flow diagram of the different groups. Group One was designated for patients that had previously diagnosed peanut allergies and had agreed to participate in a peanut challenge. Group Two patients had positive SPT testing and at least one objective symptom after ingestion of peanut. Group Three patients had positive SPT testing but had never been exposed to peanuts because of

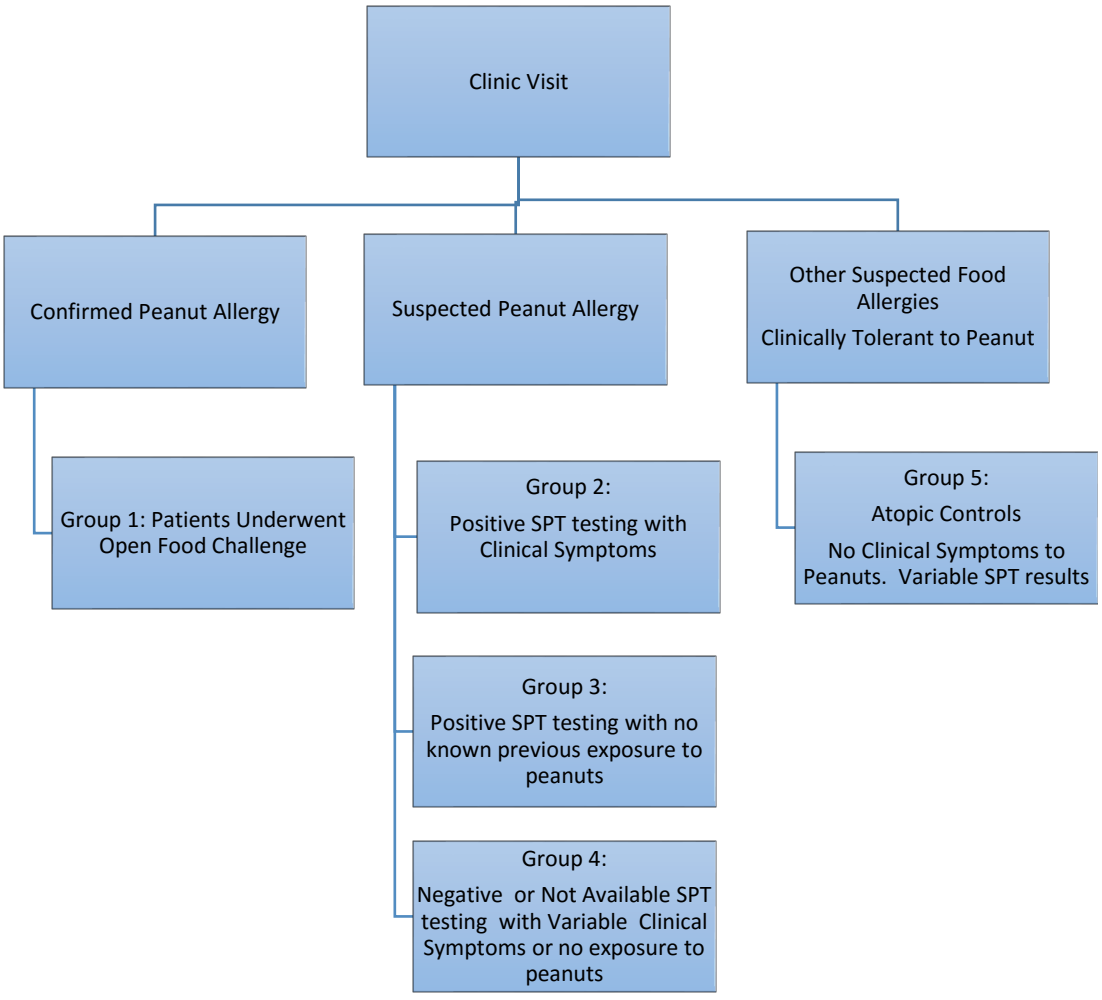


Figure 2.1 Patient Recruitment Set-up

parental preference, other family members were allergic to peanuts, or they had a strong, confirmed tree nut allergy. Group Four patients had either a negative SPT or an SPT that was not done due to a variety of reasons including risk of anaphylaxis.

## Experimental Design

One extra EDTA tube was drawn at PCH in addition to tubes patients were having drawn for routine clinical tests. The EDTA tube was then sent to ARUP where it was prepared for BAT testing. The optimal testing time was to have samples prepared and analyzed within 9.5 hours of the collection time. This was achieved in 94.5 % of patient samples. Following completion of the BAT testing, samples were spun down at 1500 rpm for 7 minutes and the plasma was then removed and stored at -20˚C in case further serological testing was needed. In most cases, the IgE testing was ordered by the physician as part of the patient's clinical labs and in cases where it was not serology testing was done after BAT was completed. The background and methodology of the SIgE and BAT tests are discussed in detail in the next two chapters.

# CHAPTER 3

# SPECIFIC IMMUNOGLOBULIN E PATTERNS TO PEANUT COMPONENTS

## Introduction to Immunoglobulin E Utility

There are five different classes of immunoglobulins: G, A, M, D, and E. The Immunoglobulin E (IgE) class was discovered in 1967 and was formally recognized by the World Health Organization International Reference Centre in 1968. It usually has the lowest concentration in the blood and has a shorter half-life then all of the other immunoglobulins.[91]

IgE is produced when the body identifies a foreign antigen such as a food or a helminth and correspondingly the levels of IgE are elevated in allergies and parasitical infections. The early IgE response is mounted in extrafollicular sources and is of low affinity. Affinity maturation and progression to the germinal center results in higher affinity IgE followed by the generation of long-lived plasma cells that sustain the IgE response.[91]

IgE can be found circulating in the blood or bound onto the surface of cells. The IgE molecules are bound onto cells via a cell receptor that bind to the Fc region of the antibody. These receptors are denoted as FcεR and there are several different classes. Class I (FcεRI) are high affinity receptors and can be found most notably on mast cells

and basophils. Class II (FcεRII) are low affinity receptors that are seen on B-cells, monocyte, and dendritic cells; however, their role in allergies is still being investigated.[92] Conventional blood tests measure the free serum IgE level. It has been shown that the serum IgE level correlates well with cell-bound IgE in most patients.[93]

It has been shown that the more IgE epitopes that a person recognizes for a specific antigen the more sensitive they are to that antigen.[94]

## Materials and Methods

All specific IgE levels were determined on an ImmunoCAP1000 instrument (ThermoFisher Scientific/Phadia) using fluorescent enzymatic immunoassay methodology. Appropriate positive and negative manufacturer controls were run alongside patients. SIgE levels were quantified down to the 0.10 kU/l level and up to the 100 kU/l level. SIgE levels of ≥0.10 kU/l were considered positive for the whole peanut and for the purified peanut components.

## Correlation of Component Testing

The correlation and reliability between whole peanut and its components on the ImmunoCAP testing platform was evaluated before the study began. SIgE data from 2,000 previous tested samples from suspected peanut-allergic patients from October 2014 to July 2015 were pulled and analyzed. Of the 2,000 results, 239 (11.95%) proved to be negative to both whole peanut and the components and were excluded from calculations. The remaining samples were then classified as to which tests were positive. Thirty-seven separate patterns of reactivity were predicted and all patterns were seen. The

patterns are shown in Table 3.1 along with the observed number of events with monosensitization patterns highlighted in light blue and di-sensitization patterns in a darker blue.

Two different types of inconclusive results were noted: whole peanut inconclusive (WPI) and peanut component inconclusive (PCI). WPI was classified as results where there was detectable whole peanut antibody levels, but all of the tested components had undetectable levels. This occurred in 146 (8.29%) of samples and the range of results varied from 0.10-9.76 kU/L. These samples could be sensitized to some of the untested peanut components, such as Ara h 5, 6, 7, 10-13, that have not been studied as thoroughly and are not commercially available. It is also possible that these samples are false positives due to cross-reactivity or lab error; however, a European study published last year did note WPI in their study and that 7 patients out of their 68 patient population (10.3%) had detectable peanut SIgE but no IgE to Ara h 1, Ara h 2, Ara h 3, Ara h 6, Ara h 8, and Ara h 9 which is similar to what was seen with our analyzed test results.[24]

PCI was classified as results where any one of the components was positive but the whole peanut extract was negative. These were of special interest as clinicians that only ordered whole peanut SIgE testing could miss these cases of sensitization. PCI occurred in 18 (1.04%) of samples with Ara h2 being the most often seen ($n$ = 8), followed by Ara h 2 and 9 ($n$ = 3, each), and then Ara h 1 and 3 ($n$ = 2, each). The range of results varied depending on the component; they are shown in Table 3.2. Most of the PCI cases had rather low values of sensitization; however, there was one concerning case (# 1021) that had a Ara h 2 value of 0.39 and no detectable response to whole peanut. As was noted before, when using an Ara h 2 cut-off point of 0.35 kU/L, there was a test

Table 3.1 Patterns of Reactivity

| | Observed Pattern | | | | | | | Total # Pts | % of Reactive | # of Pt w/>100 Results | % of Pt w/>100 Results |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Peanut | h1 | | | | | h1 only | 28 | 1.59% | 0 | 0.00% |
| 2 | Peanut | h1 | h2 | | | | h1,h2 | 143 | 8.12% | 1 | 0.70% |
| 3 | Peanut | h1 | h2 | h3 | | | h1,h2,h3 | 346 | 19.65% | 109 | 31.50% |
| 4 | Peanut | h1 | h2 | h3 | h8 | | h1,h2,h3,h8 | 98 | 5.57% | 38 | 38.78% |
| 5 | Peanut | h1 | h2 | h3 | h8 | h9 | h1,h2,h3,h8,h9 | 164 | 9.31% | 66 | 40.24% |
| 6 | Peanut | h1 | h2 | h3 | | h9 | h1,h2,h3,h9 | 106 | 6.02% | 32 | 30.19% |
| 7 | Peanut | h1 | h2 | | h8 | | h1,h2,h8 | 19 | 1.08% | 0 | 0.00% |
| 8 | Peanut | h1 | h2 | | h8 | h9 | h1,h2,h8,h9 | 6 | 0.34% | 0 | 0.00% |
| 9 | Peanut | h1 | h2 | | | h9 | h1,h2,h9 | 19 | 1.08% | 0 | 0.00% |
| 10 | Peanut | h1 | | h3 | | | h1,h3 | 21 | 1.19% | 0 | 0.00% |
| 11 | Peanut | h1 | | h3 | h8 | | h1,h3,h8 | 3 | 0.17% | 0 | 0.00% |
| 12 | Peanut | h1 | | h3 | h8 | h9 | h1,h3,h8,h9 | 5 | 0.28% | 0 | 0.00% |
| 13 | Peanut | h1 | | h3 | | h9 | h1,h3,h9 | 18 | 1.02% | 0 | 0.00% |
| 14 | Peanut | h1 | | | h8 | | h1,h8 | 1 | 0.06% | 0 | 0.00% |
| 15 | Peanut | h1 | | | h8 | h9 | h1,h8,h9 | 1 | 0.06% | 0 | 0.00% |
| 16 | Peanut | h1 | | | | h9 | h1,h9 | 1 | 0.06% | 0 | 0.00% |
| 17 | Peanut | | h2 | | | | h2 only | 264 | 14.99% | 0 | 0.00% |
| 18 | Peanut | | h2 | h3 | | | h2,h3 | 34 | 1.93% | 0 | 0.00% |
| 19 | Peanut | | h2 | h3 | h8 | | h2,h3,h8 | 13 | 0.74% | 0 | 0.00% |
| 20 | Peanut | | h2 | h3 | h8 | h9 | h2,h3,h8,h9 | 9 | 0.51% | 0 | 0.00% |
| 21 | Peanut | | h2 | h3 | | h9 | h2,h3,h9 | 15 | 0.85% | 0 | 0.00% |
| 22 | Peanut | | h2 | | h8 | | h2,h8 | 56 | 3.18% | 0 | 0.00% |
| 23 | Peanut | | h2 | | h8 | h9 | h2,h8,h9 | 20 | 1.14% | 1 | 5.00% |
| 24 | Peanut | | h2 | | | h9 | h2,h9 | 37 | 2.10% | 0 | 0.00% |
| 25 | Peanut | | | h3 | | | h3 only | 27 | 1.53% | 0 | 0.00% |
| 26 | Peanut | | | h3 | h8 | | h3,h8 | 5 | 0.28% | 0 | 0.00% |
| 27 | Peanut | | | h3 | h8 | h9 | h3,h8,h9 | 2 | 0.11% | 0 | 0.00% |
| 28 | Peanut | | | h3 | | h9 | h3,h9 | 12 | 0.68% | 0 | 0.00% |
| 29 | Peanut | | | | h8 | | h8 only | 64 | 3.63% | 1 | 1.56% |
| 30 | Peanut | | | | h8 | h9 | h8,h9 | 17 | 0.97% | 0 | 0.00% |
| 31 | Peanut | | | | | h9 | h9 only | 43 | 2.44% | 0 | 0.00% |
| 32 | | h1 | | | | | Inconclusive h1 | 2 | 0.11% | 0 | 0.00% |
| 33 | | | h2 | | | | Inconclusive h2 | 3 | 0.17% | 0 | 0.00% |
| 34 | | | | h3 | | | Inconclusive h3 | 2 | 0.11% | 0 | 0.00% |
| 35 | | | | | h8 | | Inconclusive h8 | 8 | 0.45% | 0 | 0.00% |
| 36 | | | | | | h9 | Inconclusive h9 | 3 | 0.17% | 0 | 0.00% |
| 37 | Peanut | | | | | | Inconclusive WP | 146 | 8.29% | 0 | 0.00% |

Table 3.2 Peanut Component Inconclusive Results

| ID # | Peanut | Ara h 1 | Ara h 2 | Ara h 3 | Ara h 8 | Ara h 9 |
|---|---|---|---|---|---|---|
| 176 | <0.10 | 0.11 | <0.10 | <0.10 | <0.10 | <0.10 |
| 465 | <0.10 | 0.12 | <0.10 | <0.10 | <0.10 | <0.10 |
| 368 | <0.10 | <0.10 | 0.11 | <0.10 | <0.10 | <0.10 |
| 1021 | <0.10 | <0.10 | 0.39 | <0.10 | <0.10 | <0.10 |
| 1596 | <0.10 | <0.10 | 0.10 | <0.10 | <0.10 | <0.10 |
| 347 | <0.10 | <0.10 | <0.10 | 0.15 | <0.10 | <0.10 |
| 694 | <0.10 | <0.10 | <0.10 | 0.17 | <0.10 | <0.10 |
| 231 | <0.10 | <0.10 | <0.10 | <0.10 | 0.40 | <0.10 |
| 235 | <0.10 | <0.10 | <0.10 | <0.10 | 0.22 | <0.10 |
| 591 | <0.10 | <0.10 | <0.10 | <0.10 | 0.47 | <0.10 |
| 617 | <0.10 | <0.10 | <0.10 | <0.10 | 0.46 | <0.10 |
| 949 | <0.10 | <0.10 | <0.10 | <0.10 | 0.19 | <0.10 |
| 1158 | <0.10 | <0.10 | <0.10 | <0.10 | 1.06 | <0.10 |
| 1482 | <0.10 | <0.10 | <0.10 | <0.10 | 0.16 | <0.10 |
| 1687 | <0.10 | <0.10 | <0.10 | <0.10 | 1.40 | <0.10 |
| 166 | <0.10 | <0.10 | <0.10 | <0.10 | <0.10 | 0.19 |
| 1072 | <0.10 | <0.10 | <0.10 | <0.10 | <0.10 | 0.39 |
| 1384 | <0.10 | <0.10 | <0.10 | <0.10 | <0.10 | 0.16 |

specificity of 96% and a sensitivity of 100%.[15] The high number of Ara h 8 PCI may be due to its low content in whole peanut extract.

## SIgE Sensitization Patterns

Overall, monosensitization was seen in 24.24% of the cases with Ara h 2 being the most common (15.05%) followed by Ara h 8 (3.63%), Ara h 9 (2.44%), Ara h 1 (1.59%), and Ara h 3(1.53%). This differs somewhat from the literature in that most literature has reported monosensitization as a rarer event.

Ara h 8 monosensitization has been reported with occurrence rates of 3.3%, 11.4%, 12.0%, and 17.6 % in true peanut-allergy populations in United States, Sweden, Austria, and a second Sweden population, respectively.[20,95,96] Ara h 8 and h 9

monosensitization is also well documented among peanut-tolerant patient populations.[24] Ara h 2 monosensitization (6.7%, 8.6%, 4.6%, 4.1%) was also noted by those same researchers in peanut allergic patients, but they were usually at lower rate than the Ara h 8 monosensitization.

Ara h 9 was noted in only two of the twelve studies. One had an extremely low rate of 1.4%, whereas the other study reported a rate of 36%; however, the second study population was in Spain where Ara h 9 sensitization has been reported to be higher.[20,95] Ara h 1 monosensitization was likewise reported in only two out of the twelve studies with rates of 3.3% and 6.0%.[95]

It is important to note that the different studies did use slightly different cut-off values for establishing positivity. Traditionally, 0.35 kU/l has been used as the cut-off for determining allergy for any food but since the advent of highly purified components and given the severity of peanut allergies any detectable level (>0.10kU/l) of antibodies is now being labeled as positive in most instances.

We also used our data to evaluate the correlations and patterns between the different component tests. Most literature has reported that positive results to Ara h 6 almost always occurs with a positive Ara h 2 level and that a similar relationship is seen with Ara h 1 and Ara h 2 and then Ara h 3 and Ara h 2.[96]

A statistical analysis was done on the 1,761 results with detectable IgE antibody present and scatter plots were done that show the relationship between peanut and the different tests and then between the tests themselves. Values of <0.10 were entered in as 0.09 and values of >100 were entered in as 101. Figure 3.1 shows the different scatter plots between peanut and the component tests and Figure 3.2 shows the different scatter

plots between the component tests. Ara h 2 correlated the best with the whole peanut result ($r$ = 0.92), which matches what is seen in the literature. The worst correlations were seen with Ara h 8 and Ara h 9 ($r$ = 0.04 to 0.11) which also matches the literature and is attributed to cross-reactivity to Bet v2 and lipid transfer proteins. Table 3.3 shows the correlation coefficients for the each of the SIgE test in relation to the other tests. The best correlations among the component comparisons were with Ara h 1 to Ara h 3 and h2. The correlation coefficients were fairly high, $r$ = 0.81 and 0.70, for Ara h 1 compared to Ara h 2 and Ara h 3. Ara h 3 did not correlate very well with Ara h 2, $r$ = 0.59. The analysis did show, however, that matched sensitization between Ara h 1 and Ara h 2 occurred in 92.0% of Ara h 1 sensitizations, Ara h 3sensitization occurred in 77% of cases of Ara h 1 sensitization, and Ara h 2 was present in 89.4% of cases of Ara h 3 sensitization. So although the levels of antigen did not correlate, the positivity (defined as >0.10 kU/l) did match better.

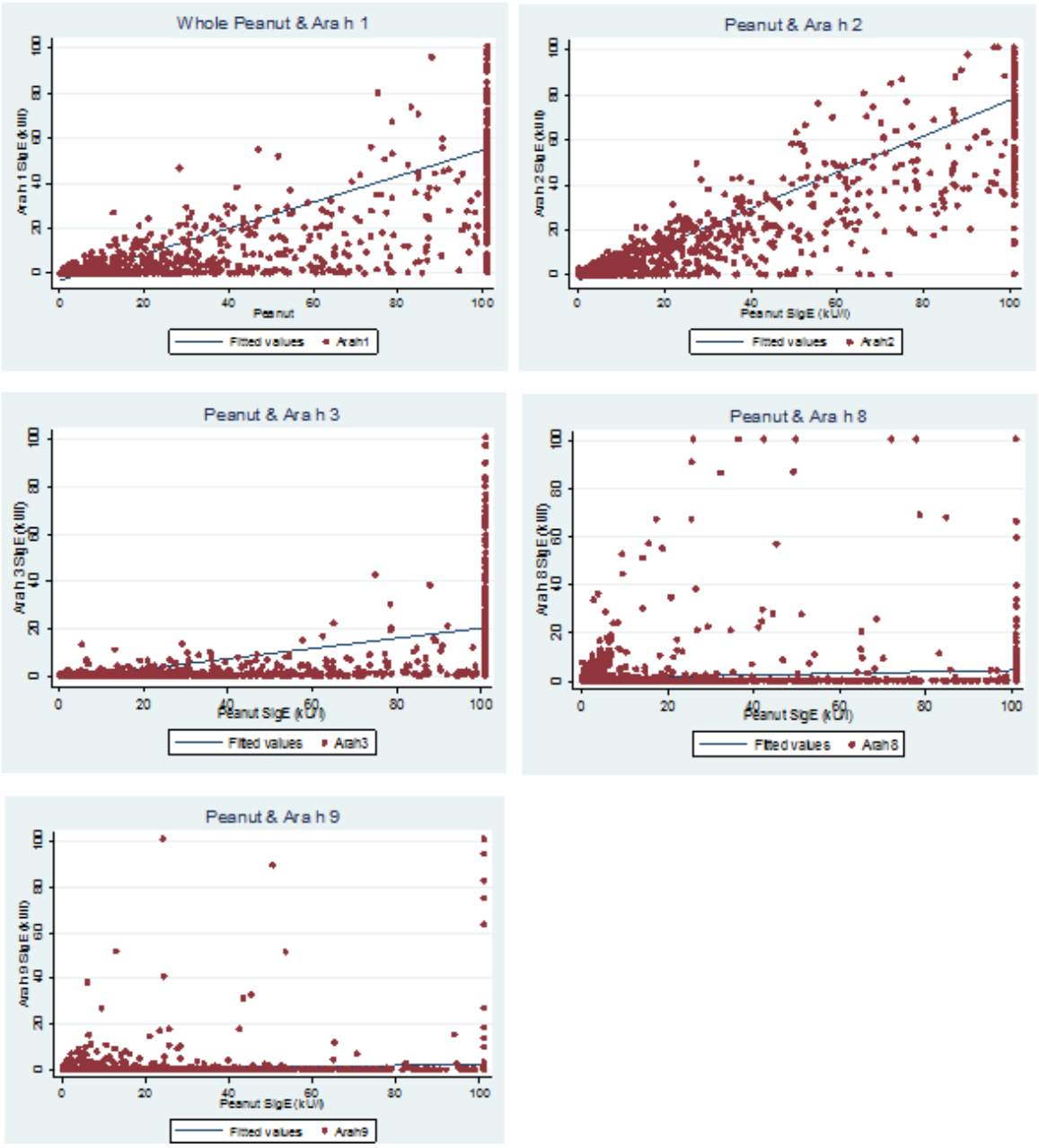


Figure 3.1 Correlation of Whole Peanut and Component SIgE

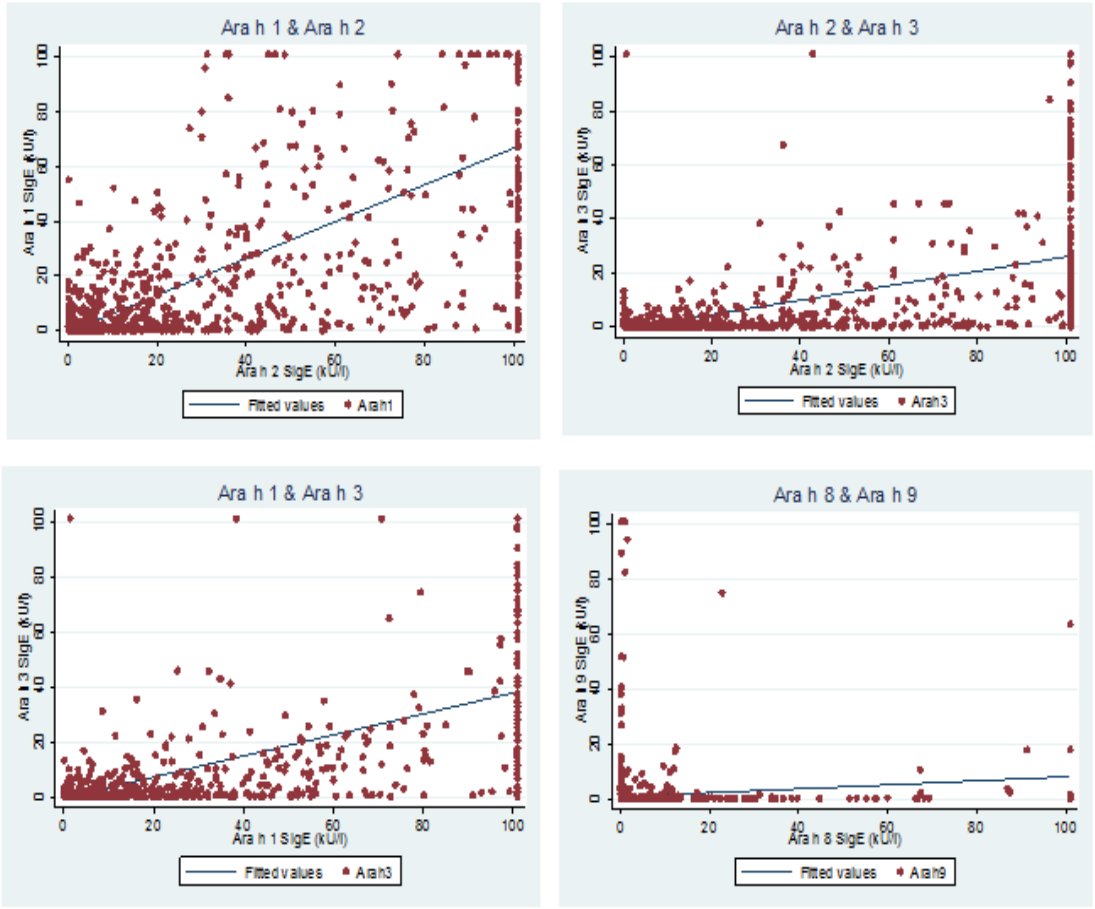


Figure 3.2 Correlation of Component SIgE Tests

Table 3.3 Correlation between SIgE Tests

| | **Peanut** | **Ara h 1** | **Ara h2** | **Ara h 3** | **Ara h 8** | **Ara h 9** |
|---|---|---|---|---|---|---|
| Peanut | 1.00 | --- | --- | --- | --- | --- |
| Ara h 1 | 0.80 | 1.00 | --- | --- | --- | --- |
| Ara h 2 | 0.92 | 0.81 | 1.00 | --- | --- | --- |
| Ara h 3 | 0.55 | 0.70 | 0.59 | 1.00 | --- | --- |
| Ara h 8 | 0.11 | 0.05 | 0.04 | 0.06 | 1.00 | --- |
| Ara h 9 | 0.10 | 0.07 | 0.05 | 0.15 | 0.11 | 1.00 |

# CHAPTER 4

# BASOPHIL ACTIVATION TESTING TO DETERMINE TRUE ALLERGY

## Introduction

There are two types of Basophil Activation Testing (BAT). The first is the simplest and is where a patient's blood is drawn and the whole blood sample is stimulated with antigen. Then, after a red blood cell lysing step, the sample is placed on a flow cytometer and gated for basophils. The second method, called passive sensitization, is where a donor provides basophils which are then stripped of all of their surface antibodies. Then patient serum is added to the stripped basophils to "reload" the surface with antibodies. The suspected antigen can then be added to see if degranulation occurs. Both methods have advantages and disadvantages. The first method is limited by the need for fresh whole blood which means samples cannot be frozen and has a preferred testing window of less than 24 hours. The second method that involves basophil-stripping eliminates the need for fresh patient whole blood and can use patient serum which can be frozen and is stable for weeks or months depending on the storage temperature. However, the second method is much harder to standardize. Different donors have different basophil responses and special care has to be taken to ensure that

the basophils are not activated while they are being stripped. This has led to interest in creating cell lines that contain the FcεRI receptor and could be loaded with patient IgE and therefore be more standardized and reliable then donor basophils.

Unfortunately many obstacles still need to be overcome. For cell lines that are not of human origin, like rat basophilic leukemia cell lines (RBL), human FcεRI receptors must be transfected into the cells. One study evaluated three different RBL cell lines and found that all of these transfected humanized cell lines had a high percentage of IgE binding to the FcεRI receptors; however, they had inconsistent degranulation when an allergen was introduced and they had loss of the expression of the human FcεRI receptors after prolonged culture.[97] It is important to note that the inconsistent degranulation may be due to several different factors. The first is that there can be many other specificities of the IgE antibodies present in a patient's serum. For example, more anti-dog, anti-pollen, or nonspecific antibodies might end up binding to the FcεRI receptors instead of the antibody of interest. The second is that when using transfected cell lines, there is a variable number of FcεRI receptors that are expressed on the cell surface. The number of receptors on the cell line may also not match the number of receptors on the patient's basophil; therefore, the cells lines may have decreased or increased sensitivity.

It is also important to avoid contamination with aeroallergens and occupational allergens that can be found in the clinical lab. Contamination from latex gloves or airborne contaminants, like molds or pollen, can cause false positives or it can modify the results in cases where a patient is truly allergic to the allergen of interest. It has been shown that at low allergen concentrations additions of a second allergen increases the

degranulation response while at higher allergen concentrations a second allergen decreases the response.[98]

## Cellular Flow Markers

Several different flow cytometry markers and parameters can be used in BAT testing, including CCR3, SSC, CD63, CD213, HLA-DR, CD203, anti-IgE, anti-FCεRI, and fMLP.

CCR3, CD123, HLA-DR, IgE, and SSC are used to identify the basophil population. CCR3 (eotaxin receptor) is expressed on both basophils and eosinophils. It has been shown to be a stable and well expressed marker of basophils.[99] A slight decrease in CCR3 expression upon basophil activation has been noted. Some studies noted a 40% gMFI reduction in CCR3 expression and that it displayed adequate sensitivity but weak specificity when used as a basophil activation marker.[100] *Hausmann et al* evaluated this decreased expression in terms of BAT testing and found that the relative number of acquired stimulated basophils did not reach statistical significance and that it was the most consistent marker in all of their research donors.[99] It is important to note that CCR3 is not lineage specific and can be expressed on other cells so secondary parameters must be used in conjunction with it.

CD123 is an interleukin 3 receptor and can be found on many subsets in the peripheral blood including basophils, eosinophils, monocytes, and dendritic cells. It can be used along with HLA-DR to select for basophils ($CD123^{high}$/$HLA\text{-}DR^{neg}$). It has been shown that the CD123 and HLA-DR levels do not significantly change with basophil activation.[101]CD123 has been found to have a more variable expression on basophils than

the more commonly used CCR3 and inflammation can cause HLA-DR expression to change.[99]

IgE is problematic as a basophil identification marker. Although basophils do have bound IgE molecules on their surface, there is great variation between individuals which is dependent on their IgE plasma levels. In certain donors, up to 80% of cells in the basophil gating parameters would be misidentified if anti-IgE markers were used.[99]

CD63 is also known as the lysosome-associated membrane protein-3, and as previously mentioned, it is an activation marker that is widely used in BAT testing either by itself or in conjunction with CD203. CD63 has an extremely low expression rate on resting basophils and therefore has high specificity for basophil activation.[102] CD63 upregulation occurs within a minute after stimulation with antigen and optimal expression of the marker occurs after 15-30 minutes.[101] CD63 expression has also been linked more closely to anaphylactic degranulation, fast morphological changes, and release of intracellular granules than other markers like CD203.[102] It has also been noted that CD63 has a bimodal expression with dim and bright expression on basophils,[101,102] but the clinical significance of this bimodal expression has yet to be investigated. CD203, also known as ecto-nucleotide pyrophosphatase phosphodiesterase-3, can also be used as a marker of activation because its levels are upregulated on degranulated basophils by several factors. When using fMLP or anti-IgE controls, the upregulated tends to increase by a factor of 2-3.[101] Since CD203 is present on resting basophils, however, researchers must be careful when using it and its upregulation is not as significant as CD63.[102]

## Materials and Methods

For this research study, we used the FlowCAST kit manufactured by Buhlmann Laboratories. The manufacturer's recommended protocol was used and the whole peanut extract, Ara h 1, and Ara h 2 allergens were also obtained from the manufacturer. Recombinant Ara h 8 antigen (RP-AH8-1) was obtained from Indoor Biotechnologies and was run using the same protocol as the whole peanut extract. Fifty microliters of patient whole blood was added to 100ul of a stimulation buffer that contained IL-3 to help prime the basophils and calcium to replace the calcium that was bound by EDTA. Fifty microliters of the peanut antigen was also added to each tube. Three different concentrations were examined at for each allergen resulting in a dose-response curve. Whole peanut and Ara h 8 antigens were performed at 100ng/ml, 20ng/ml and 4 ng/ml. Then as per the manufacturer, Ara h 1 was run at 1000ng/ml, 200ng/ml, and 40 ng/ml and Ara h 2 was run at 200ng/ml, 40ng/ml, and 0.8ng/ml. Twenty microliters of anti-CD63-FITC and anti-CCR33-PE mouse antibodies, used to identify the basophils and the % of activation, were added followed by a 15-minute incubation in a 37˚C water bath. After incubation, 2 milliliters of lysing reagent was used to remove RBC's and a quick wash step was used to remove cell debri. The remaining cells were then run on a BD Canto flow cytometer. A total of fifteen flow assays were performed on each patient: a background/negative control, two positive controls, three concentrations of whole peanut antigen, three concentrations of Ara h 1, three concentrations of Ara h 2, and three concentrations of Ara h 8.

The background/control contained only stimulation buffer. A base value of less than 2.0-2.5% activated basophils was previously determined to be acceptable as a

negative control by the kit manufacturer. There were two positive controls that were defined as acceptable if one control caused >10% of basophils to upregulate CD63. The first positive control that was used was anti-FcεRI mouse antibodies that causes cross-linking of surface-bound IgE antibodies similar to the allergen. The second positive control was a formyl-methionyl-leucyl-phenylalanine (fMLP) peptide which is produced during bacterial growth. The fMLP causes basophil activation by binding to cell surface receptors that act through G proteins and phospholipase C. It has been used as a stimulus for neutrophil, monocyte, and basophil activation.

Basophils were defined as the CCR3pos/SSClow population. The percentage of basophils that displayed CD63 was calculated. Positive allergic responses were defined as ≥10% upregulation of CD63. See Figure 4.1 for a typical gating strategy on a patient. A total of 500-600 basophils was the preferred range for analysis and this was achieved in the majority of results (82%) and analysis of the background test for each patient showed the mean basophil recovery was approximately 558. As per the manufacturer, patients with less than 200 basophils recovered were not eligible for analysis. No patient results had to be excluded from this study due to low recovery.

We attempted to include all of the tested SIgE peanut components in the BAT testing; however, Ara h 3 and Ara h 9 were not commercially available.

## Nonresponders and Signal Transduction

One major drawback of BAT is that a significant proportion of the population have basophils that do not respond to stimulation with anti-IgE or anti-FcεRI. These patients are called "nonresponders." Studies have reported nonresponder rates between

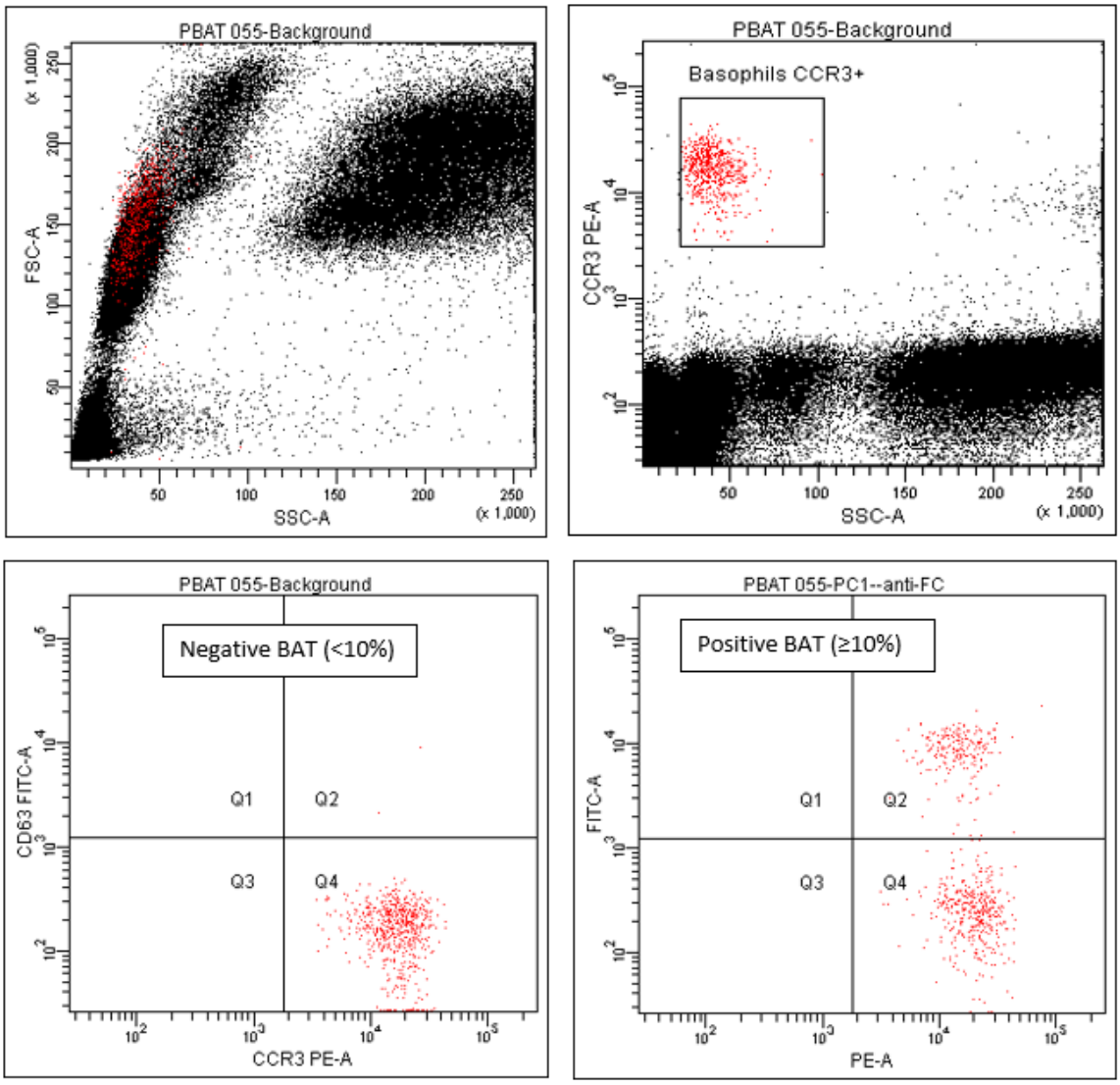


Figure 4.1 Representative BAT Gating Strategy

11.5-22%.[103,104,105] Nonresponsive basophils do not appear to have any mutations or structural changes in their FcεRI.[106] In addition, they have similar cellular densities of IgE antibody as responsive basophils and can be activated by other non-IgE pathway through the use of different reagents such as fMLP, calcium ionophore A23187, and 12-0-tetradecanoyl phorbol-13 acetate.[105] It is currently believed that nonresponding basophils have issues with components in the intracellular signal transduction pathway. It has been shown that nonresponding basophils have hardly any changes in cytosolic free $Ca^{2+}$ after the addition of anti-IgE and when given a second challenge with a non-IgE stimulus the response is inhibited, which is indicative that intracellular signals were generated but there was a problem somewhere in the signal cascade.[107]

The initial cross-linking and activation of the FcεRI receptors leads to the activation of Lyn, a protein tyrosine kinase, which in turn phosphorylates the immunoreceptor tyrosine-based activation motifs on the β and γ subunits of the receptor and then Syk is recruited and activated. Eventually, the cascade leads to the creation of second messengers, IP3 and 1,2-diacylglycerol in particular, that cause the release of $Ca^{2+}$ and start to trigger the release of basophilic granules and histamine.

It has been shown that two regions in the IgE-mediated signal transduction pathway are downregulated during stimulation: one that controls the protein kinase Syk's phosphorylation, and one that controls the PI3 Kinase.[108] The Syk kinase in particular has been shown to decrease by 20% after a 1-hour stimulation and an 80% decrease in incubations >18 hours.[109] This appears to be due to ubiquitination of syk by c-cbl, which is originally phosphorylated and activated by syk, lyn or fyn, and it has been suggested that low cytosolic calcium may also enhance syk ubiquitination.[109] The loss of syk was

also looked at in a small study that took purified nonresponding basophils from 3 different donors and performed Western blots to look at the levels of syk and lyn. It was found that the basophils failed to express syk and had normal to reduced levels of Lyn.[110]

Interleukin 3 (IL-3) has been shown to restore the signaling function of non-responding basophils.[106] A 3-day incubation period with IL3, however, is needed to restore functionality. This makes the use of IL-3 in clinical testing problematic and calls into question how the results would correlate in vivo. A short 15-minute incubation with IL-3 buffer was used in our assay, but its main function was to help prime basophils and not address the nonresponder issue. It has been shown that IL-3 can increase the sensitivity of basophils when in the presence of certain antigens and can help provide a better distinction between antigen-stimulation activation and unspecific activation.[102]

## Basophil Allergen Thresholds: CD-sens and CD-max

CD-sens is defined as the allergen concentration that causes 50% of basophils to activate and upregulate CD63 and it is measured by inverting the allergen concentration and multiplying by 100.[55] CD-max is defined as the maximal percentage of CD63 upregulation at any one allergen dose[90] which can vary from person to person, in part, due to the variability of bound IgE which is necessary to form crosslinking. CD-sens has been shown to be a better measure of allergen sensitivity then CD-max.[90] It has also been used in conjunction with food challenges and it has been found that 92% of children that fail a food challenge have positive CD-sens.[111]

The optimal cut-off concentration for discriminating between peanut-allergic and peanut-sensitized patients has been established as 100ng/ml.[104] The 100ng/ml cut-off for

peanut was used as our highest concentration in this study, which was in line with manufacturer recommendations. The manufacturer also recommended testing four other concentrations, but we only did two additional concentrations since the aim of our study was to correctly diagnose peanut allergies rather than monitor desensitization therapies. The three concentrations do allow us to get a sense of the severity of the allergen and provide a CD-sens in all but the extremely sensitive peanut allergy patients that react at very small amounts of antigen.

# CHAPTER 5

# RESULTS

## PCH Patient Results

Fifty-four total patients were recruited from PCH; however, 13 patients had to be excluded from the analysis. Nine patients (16.6%) were determined to be non-responders, 8 patients from the peanut allergy group and 1 from the atopic controls. Four patients also had to be excluded due to high backgrounds. At least 3 of these high background patients were linked to a newly opened bottle of anti-CD63 reagent that was suspected of having bacterial contamination. This left us with a total of 35 peanut allergy patients and 6 controls.

SIgE positivity in the peanut allergy patients was 94.3% to whole peanut extract, 54.3% to Ara h 1, 68.6% to Ara h2, 54.3% to Ara h 3, 17.1% to Ara h 8, and 14.3% to Ara h 9. Monosensitization to a single component was seen to Ara h 2 (14%), Ara h 3 (8.5%), and 1 patient who had Ara h 8 monosensitization and, after a peanut challenge, was proven to be tolerant to peanut.

The PCH patients were originally divided into five groups, as was outlined in the Patient Recruitment section, based off of skin prick testing (SPT) results and clinical symptoms. Group 1 was of special interest in that they were patients that had been

previously diagnosed with peanut allergy and had consented to undergo a food challenge. There were 3 patients in this group. Two out of the 3 had detectable SIgE levels to peanut (>100, 0.22 kU/l) and the third was monosensensitized to Ara h 8 (0.41 kU/l). They all had convincing clinical histories of peanut allergies. The BAT results in these cases did predict the outcome of the food challenge.

The first patient was a 19-year-old female that had an IgE serum level of >100kU/L to whole peanut, Ara h 1, and Ara h 2. She had been undergoing treatment with Xolair and her peanut SPT results had decreased from 38 to 13 to 0. The BAT results showed no reactivity at the lowest concentrations of allergen, but there was basophil activation at the higher concentrations for all three allergens: 100ng/ml peanut = 60.7% activation, 20ng/ml = 14.8% activation, 1000ng/ml Ara h 1 = 17.5% activation, 20 ng/ml Ara h 2 = 51.3% activation, 4ng/ml = 14.7% activation. The patient was able to tolerate 4mg of peanut protein before hives developed and the oral challenge was ended. She was put on an OIT regimen for several days but developed severe abdominal pain and that too was discontinued.

In the two other food challenges, the BAT results for all the tested allergens were negative (<10% activation) and there was only a minor sensitization to Ara h 3 (0.13 kU/l) in one of the patients. The Ara h 8 monosensitized patient in Group 1 was unique and the tolerant status of the patient matches the current literature of Ara h 8 positive patients having more mild or negligible symptoms. Studies using Ara h 8 in BATs were looked for when we started this project as there were none published; however, one paper published during our project that looked at basophil threshold sensitivity's in monosensitized Ara h 8 children found that 17 out of 20 children reacted to Ara h 8 at

varying concentrations (1.2-82.8 ng/ml).[112] This study seems to suggest that Ara h 8 might be still be an important allergen in these monosensitized populations. Unfortunately, we only had the 1 tolerant Ara h 8 monosensitized patient so we could not evaluate the importance of Ara h 8 in a true monosensitized allergic patient population. We did have 7 patients with detectable IgE to Ara h 8, 6 patients in the peanut allergy group and 1 patient in the control group. The median value of Ara h 8 in the peanut allergy group was 0.97 kU/l and the range was 0.28-2.36 kU/l. In all cases, the patient basophils did not react to the Ara h 8 antigens and they all had varying degrees of sensitization to Ara h 1, Ara h 2, and Ara h 3, so it is possible Ara h 8 is not as important in patients with multiple peanut component sensitizations. There are also two isoforms of Ara h 8: 8.0101 and 8.0201.[33] We only used 8.0101 in our research study. The previously mentioned study did not note which isoform they used so it is also possible there might be a difference between the isoforms.

The different PCH groups were evaluated to see if there was a difference in the nonresponder rate, total IgE, individual SIgE levels, and BAT upregulation at the highest concentration of each of the allergens. The information is summarized in Table 5.1 and Figure 5.1 illustrates the mean difference in BAT upregulation for the whole peanut antigen and displays calculated *p*-values (*t*-test). The rate of nonresponders was found to be pretty evenly distributed with 1 to 3 nonresponders occurring in each group. The mean total IgE was generally around 300kU/l though it was a little higher in Group 4 (582.1 kU/l), this group had a 'not available' SPT which occurred frequently when the patient had extremely high levels of SIgE and had known anaphylaxis to peanut such that physicians did not feel that an SPT was warranted. There were a few overall trends. In

Table 5.1 PCH Patient Results by Group

| | **Group 1 Peanut Challenge** | **Group 2 SPT pos. w/ Symptoms** | **Group 3 SPT pos No exposure** | **Group 4 n/a SPT variable symptoms** | **Group 5 Controls** |
|---|---|---|---|---|---|
| # of Patients | 4 | 19 | 10 | 11 | 7 |
| # of Non-responders | 1 | 2 | 2 | 3 | 1 |
| Mean TIgE (kU/L) | 337.9 | 340.4 | 258.1 | 582.1 | 368.7 |
| *Ranges* | *(9.62-900)* | *(6.7-1271)* | *(59-697)* | *(12-1613)* | *(22-1899)* |
| **Whole Peanut** | | | | | |
| Mean SIgE (kU/L) | 33.7 | 23.2 | 12.8 | 36.3 | 3.6 |
| *Ranges* | *(0-101)* | *(0.10-101)* | *(0.57-45.2)* | *(0-101)* | *(0-21.7)* |
| Mean BAT % | 20.7 | 29.3 | 36.9 | 40.8 | 0.45 |
| *Ranges* | *(0.2-60.7)* | *(0-91.2)* | *(0.2-84.2)* | *(0.2-90.5)* | *(0-1.5)* |
| **Ara h 1** | | | | | |
| Mean SIgE (kU/L) | 33.7 | 11.4 | 2.0 | 24.9 | 0.05 |
| *Ranges* | *(0-101)* | *(0-73.7)* | *(0-9.22)* | *(0-101)* | *(0-0.31)* |
| Mean BAT % | 6.3 | 22.3 | 26.1 | 30.4 | 0.38 |
| *Ranges* | *(0.2-17.5)* | *(0-87.7)* | *(0.5-70.3)* | *(0.2-93.8)* | *(0.2-0.9)* |
| **Ara h 2** | | | | | |
| Mean SIgE (kU/L) | 33.7 | 17.3 | 8.14 | 32.4 | 0.01 |
| *Ranges* | *(0-101)* | *(0-101)* | *(0-23.1)* | *(0-101)* | *(0-0.04)* |
| Mean BAT % | 17.6 | 22.2 | 32.9 | 34.6 | 0.12 |
| *Ranges* | *(0.2-51.3)* | *(0-88.4)* | *(0.1-71.8)* | *(0-87)* | *(0-0.7)* |
| **Ara h 8** | | | | | |
| Mean SIgE (kU/L) | 0.14 | 0.10 | 0.30 | 0.20 | 0.21 |
| *Ranges* | *(0-0.41)* | *(0-1.23)* | *(0-2.36)* | *(0-1.1)* | *(0-1.26)* |
| Mean BAT % | 0.50 | 0.66 | 0.55 | 0.65 | 0.52 |
| *Ranges* | *(0-1.2)* | *(0-1.7)* | *(0.3-1.0)* | *(0-2.3)* | *(0-1.5)* |

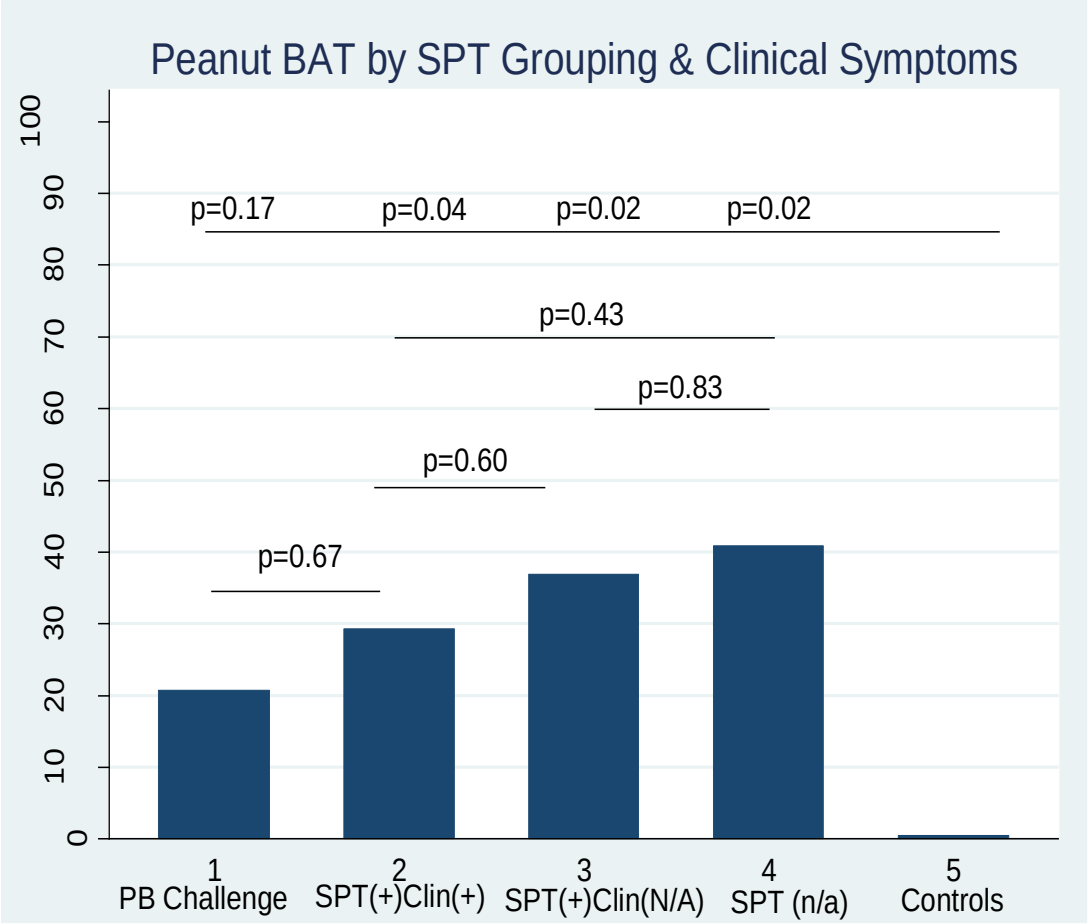


Figure 5.1 Analysis of Whole Peanut BAT by SPT Group

particular, Group 3 patients who had never been exposed to peanut tended to have slightly lower SIgE levels than the rest of the peanut allergy groups. There was not a statistically significant difference in the BAT between the individual groups ($p$=0.43-0.83); however, in comparison to the controls, all groups except for Group 1 (Food Challenges) had statistically significant with $p$ values ranging from 0.02 to 0.04.

We originally placed the patients into groups by using SPT and the presence of exposure and clinical symptoms in hopes of finding a testing algorithm and patient population that would greatly benefit from BAT testing. This, unfortunately, did not occur with this grouping scheme. One possible reason for this may be the heterogeneity of the allergy population and the different reasons for the same outcome and grouping, ie, a 2-year-old child whose mother never exposed them to peanut for fear of them developing an allergy and tests mildly positive would end up in the same group as a child that has anaphylaxis to tree nut and positive peanut SPT and is told by a physician to never be exposed to peanuts. Or in the case of SPT testing, large differences can be seen where one child will not have testing done because they have already had a documented anaphylaxis reaction versus another who perhaps will not behave long enough for SPT testing to be completed.

Since the original grouping system proved ineffectual, a new category evaluating severity was added for each patient and the patients were then reclassified based on their clinical presentation. Each patient was evaluated by a physician that had been blinded to the BAT results. In general, mild cases were defined as patients with cutaneous symptoms such as hives, swelling, redness, and itching. Moderate cases were defined as patients with gastrointestinal symptoms such as vomiting and nausea and with or without

cutaneous symptoms. Severe cases were defined as patients with symptoms of anaphylaxis and respiratory distress that required the use of epinephrine and hospitalization. Then, there were also two categories for those never exposed to peanut and for those who had peanut allergy but then became tolerant to peanut. The second analysis using these severity categories is summarized in Table 5.2 and illustrated in Figure 5.2 and Figure 5.3.

Using severity of symptoms, instead of SPT and the presence or absence of symptoms, showed a much clearer picture of peanut allergies. The total IgE increases steadily among the groups from 53.8, 321.4, 675, to 829 in the tolerant, mild, moderate, and severe groups, respectively.

The difference between the severity groups themselves were, in general, not statistically significant ($p = 0.21 – 0.50$)(*t*-test); however, comparison between the severe and mild symptom groups was significant with a $p$ value of 0.04. Which is a very important discovery as it implies that it may be possible to discriminate between severe and mild peanut allergies using BAT without exposing children to peanut and risking allergic reactions. There were several statistically significant results when comparing the severity groups to the negative controls. The severe, moderate, and mild symptom groups had a $p$ value of 0.0007, 0.04, and 0.03, respectively. The tolerant and not exposed symptom groups did not achieve statistically significance ($p = 0.61$ and 0.06); this may be due to the lower numbers of patients in the tolerant group ($n = 2$) and the mixed population in the ‘not exposed’ group where allergy status is somewhat questionable since they have never been exposed to peanut.

The SIgE also increased among the severity groups except in the case of the

Table 5.2 PCH Patient Results by Severity

| | Not Exposed | Tolerant | Mild | Moderate | Severe | Controls |
|---|---|---|---|---|---|---|
| # of | 11 | 2 | 13 | 4 | 5 | 6 |
| Mean TIgE | 185.9 | 56.8 | 321.4 | 675 | 829 | 368.7 |
| *Range* | *18-584* | *9.62-104* | *6.73-1090* | *18-1452* | *88-1613* | *22-1899* |
| Mean | 63.8 | 9.5 | 71.8 | 60.3 | 66 | 81.7 |
| *Range* | *22.24-* | *34.7-44.3* | *21.2-92.4* | *16.8-94.8* | *10.7-87* | *63.3-89.3* |
| **Whole Peanut** | | | | | | |
| Mean SIgE | 9.2 | 0.11 | 19.5 | 75.9 | 41.6 | 3.6 |
| *Range* | *0.12-45.2* | *0-0.22* | *0->100* | *0.63->100* | *4.38-* | *0-21.7* |
| Mean BAT | 28.4 | 0.07 | 28.1 | 40.7 | 62.2 | 0.45 |
| *Range* | *0.2-84.2* | *0.2-1.2* | *0-91.2* | *0.3-90.5* | *13.8-90* | *0-1.5* |
| **Ara h 1** | | | | | | |
| Mean SIgE | 1.13 | 0.05 | 10.8 | 58.8 | 22.4 | 0.05 |
| *Range* | *0-9.22* | *0-0.09* | *0-73.7* | *0.05->100* | *0-57.9* | *0-0.31* |
| Mean BAT | 19.3 | 0.7 | 17.0 | 35.1 | 50.6 | 0.38 |
| *Range* | *0.2-70.3* | *0.2-1.2* | *0-87.7* | *0.5-93.8* | *14.5-78.7* | *0.2-0.9* |
| **Ara h 2** | | | | | | |
| Mean SIgE | 5.85 | 0.02 | 12.8 | 75.8 | 33.6 | 0.01 |
| *Range* | *0-23.1* | *0-0.03* | *0-74* | *0.31->100* | *4.69-* | *0-0.04* |
| Mean BAT | 24.2 | 0.75 | 22.3 | 37.3 | 48.4 | 0.12 |
| *Range* | *0.1-71.8* | *0.3-1.3* | *0-88.4* | *0-87.0* | *7.2-79* | *0-0.7* |
| **Ara h 8** | | | | | | |
| Mean SIgE | 0.22 | 0.2 | 0.1 | 0.12 | 0.28 | 0.21 |
| *Range* | *0-2.36* | *0-0.41* | *0-1.23* | *0-0.46* | *0-1.1* | *0-1.26* |
| Mean BAT | 0.82 | 0.6 | 0.55 | 0.40 | 0.52 | 0.52 |
| *Range* | *0.2-2.3* | *0-1.2* | *0-1.5* | *0.3-0.7* | *0-1* | *0-1.5* |

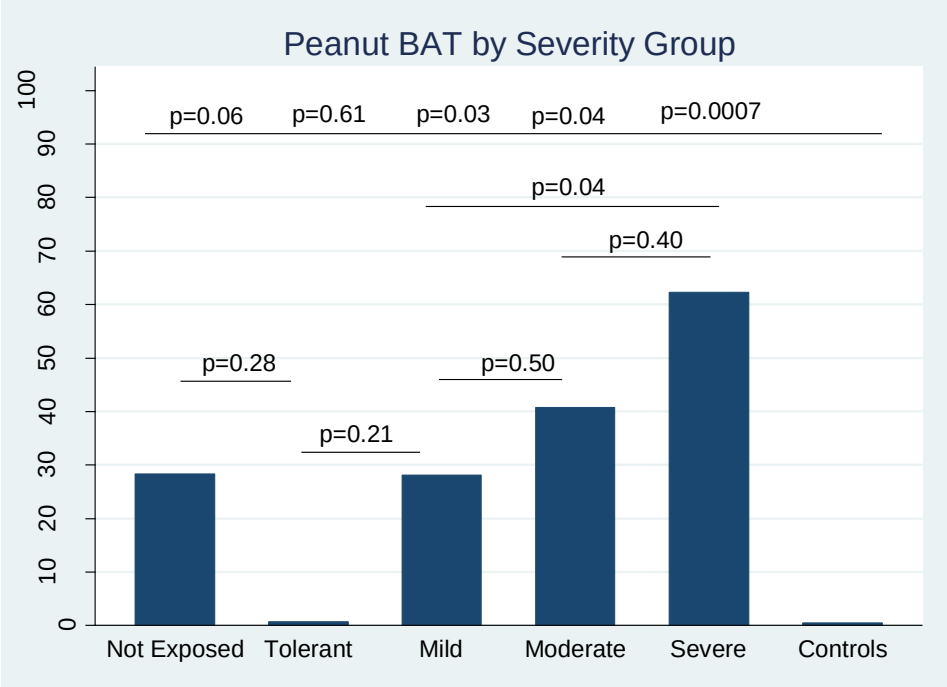


Figure 5.2 Analysis of Whole Peanut BAT by Severity Group

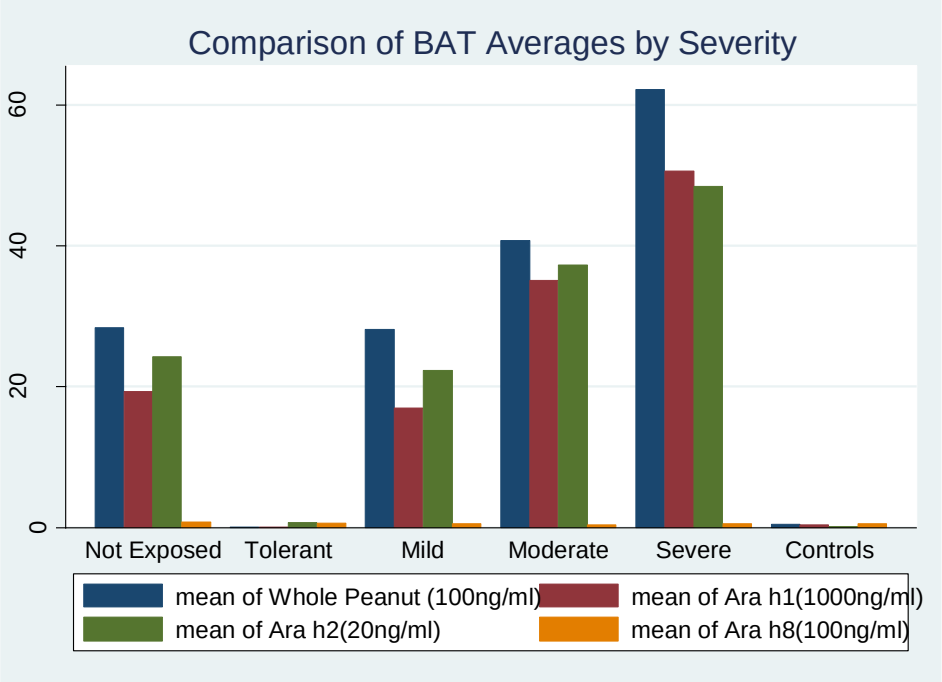


Figure 5.3 Comparison of BAT Averages by Severity Group

severe group which was actually lower than the moderate group. Further investigation of the 5 severe patients showed that 1 patient had >100 kU/l to peanut and another 3 also had significantly higher than the accepted SIgE cut-off of >15kU/l for determining a peanut allergy. The last patient, however, had much lower values of 4.38 kU/l to peanut which lowered the group mean. This patient was a 5-year-old female with a reported history of anaphylaxis to peanut, monosensitization to Ara h 2 (4.69 kU/l), and a discordant SPT value of less than 5 mm to peanut. Her BAT results were also unique in that she had a 90% activation of her basophils at 100ng/ml of peanut antigen, only 2 other patients had higher values of 90.5% and 91.2%, and she also had a positive 68.7% BAT upregulation when stimulated with 1000ng/ml of Ara h 1 antigen despite not being sensitized to Ara h 1—this was the only instance of a BAT being positive when there was no IgE sensitization in the whole study. It is unclear whether this is a valid BAT result or whether there might have been contamination during the lab test. Performing another BAT when the child comes in on her next appointment might help resolve this issue.

There was also an outlier in the moderate severity group. Three out of the 4 moderate patients had >100 kU/l, but the last patient only had 0.63 kU/l to peanut. This patient was a 1-year-old female with a clinical history of hives and respiratory problems when exposed to peanuts with a peanut SPT of 2 mm. She also showed no positivity in the BAT tests. Further investigation of her clinical history showed that she had an accidental peanut exposure a month previously that had required an Emergency Room visit. It is possible that she was in a type of anergic state following that exposure.

The BAT upregulation among the different groups increased steadily as severity increased supporting the literature claims that BATs can help improve peanut diagnosis

by helping determine the severity of the peanut allergy. The analysis of the BAT averages was done at the highest concentration of each allergen. The negative controls and tolerant group all had mean BATs of <10% which was expected and supported the high specificity of BAT for true allergies. The BAT means for whole peanut were 28.1, 40.7, and 62.2% in the mild, moderate, and severe groups. The BAT means for Ara h 1 and Ara h 2 in the same groups were 17, 35.1, 50.6, and 22.3, 37.3, and 48.4%. Since patients can have monosensitization to single components, statistical analysis of the means was not warranted for Ara h 1 and Ara h 2 and is not shown. We did look at the individual patients in each group to see if any group had a stronger BAT response despite having lower IgE levels, but there was wide variability among all the patients and within each group. The results are detailed in Table 5.3. A good example of this wide

Table 5.3 Component SIgE versus BAT by Severity Group

| | Individual Patients with SIgE Sensitization | | | | |
|---|---|---|---|---|---|
| | #1 | #2 | #3 | #4 | #5 |
| **Mild Symptoms** | | | | | |
| Ara h 1 IgE (kU/l) | | | | | |
| Ara h 1 BAT (%) | | | | | |
| Ara h 2 IgE (kU/l) | 0.27 | 0.52 | 1.34 | | |
| Ara h 2 BAT (%) | 5.2 | 15.2 | 9.7 | | |
| **Moderate Symptoms** | | | | | |
| Ara h 1 IgE (kU/l) | 33.3 | >100 | >100 | | |
| Ara h 1 BAT (%) | 28.7 | 17.5 | 93.8 | | |
| Ara h 2 IgE (kU/l) | 0.31 | >100 | >100 | >100 | |
| Ara h 2 BAT (%) | 0.2 | 11 | 51.3 | 87 | |
| **Severe Symptoms** | | | | | |
| Ara h 1 IgE (kU/l) | 6.42 | 13.6 | 33.9 | 57.9 | |
| Ara h 1 BAT (%) | 31.2 | 60.1 | 14.5 | 78.7 | |
| Ara h 2 IgE (kU/l) | 4.69 | 5.8 | 10.2 | 45.6 | >100 |
| Ara h 2 BAT (%) | 72 | 24.5 | 79 | 7.2 | 79.7 |

variability between SIgE and BAT is in the severe group which had 2 patients with ~5kU/l IgE Ara h 2 sensitizations that produced BAT results of 24.5 and 72.0%. We also looked at the overall correlation of SIgE to BAT results regardless of severity group. We felt this was important since SIgE is currently the only other serum test for allergies. An analysis of each of the component SIgE levels compared to the BAT results of that allergen were done and are summarized in Figure 5.4 and Table 5.4. There was not a strong correlation between the SIgE/BAT results for whole peanut and Ara h 2 (r ~0.5). Ara h 1 had a slightly stronger correlation (r~0.7) whereas Ara h 8 tended to have a negative correlation (r~ -0.13) This is in line with the known fact that specific IgE only measure sensitization and that there can be patients with high levels of SIgE and no actual allergy or only mild symptoms.

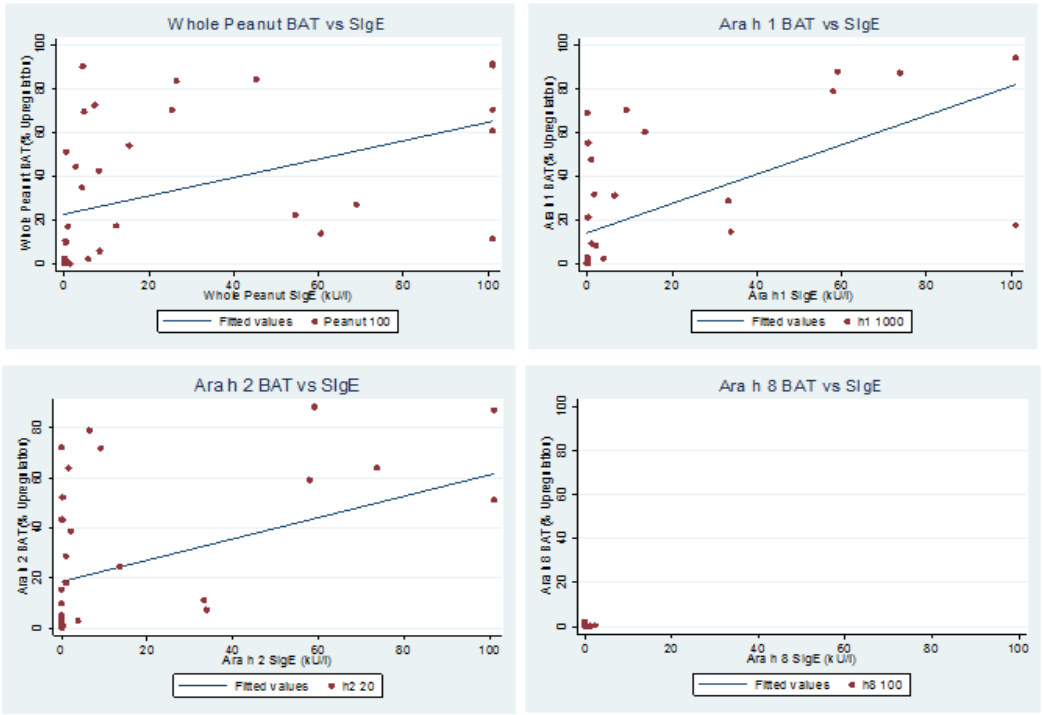


Figure 5.4 Correlation of SIgE Levels to BAT

Table 5.4 Correlation Between SIgE and BAT

| | **Total IgE** | **Peanut SIgE** | | **Ara h 2 SIgE** | **Peanut SIgE** |
|---|---|---|---|---|---|
| Peanut BAT *(100ng/ml)* | 0.54 | 0.46 | Ara h 2 BAT *(20ng/ml)* | 0.48 | 0.57 |
| Peanut BAT *(20ng/ml)* | 0.54 | 0.59 | Ara h 2 BAT *(4ng/ml)* | 0.52 | 0.55 |
| Peanut BAT *(4ng/ml)* | 0.45 | 0.49 | Ara h 2 BAT *(0.8ng/ml)* | 0.45 | 0.44 |
| | **Ara h1 IgE** | **Peanut SIgE** | | **Ara h 8 SIgE** | **Peanut SIgE** |
| Ara h 1 BAT *(1000ng/ml)* | 0.62 | 0.66 | Ara h 8 BAT *(100ng/ml)* | -0.19 | -0.18 |
| Ara h 1 BAT *(200ng/ml)* | 0.71 | 0.69 | Ara h 8 BAT *(20ng/ml)* | -0.13 | 0.12 |
| Ara h 1 BAT *(40ng/ml)* | 0.71 | 0.60 | Ara h 8 BAT *(4ng/ml)* | -0.13 | -0.09 |

Threshold levels were also found to have a marked difference between the different severity groups. Thirty-eight percent of the mild symptoms group had no response to any concentration of the whole peanut antigen. In contrast, the 75% of the moderate symptoms group patients and 100% of the severe symptoms group had reactivity to at least one concentration of whole peanut. The percentage of patients responding to all concentrations of antigen also increased across the groups from 15.3% in the mild symptoms group to 25% in the moderate symptoms group to 60.0% in the severe symptoms group, suggesting that the more severe patients would react at lower concentrations of peanuts.

Overall, there were 12 patients that had <10% activation to the whole peanut antigen and all of the other tested antigens. Two of them were classified as tolerant and had undergone peanut challenges. Four patients were classified as having mild symptoms

and 5 patients had never been exposed to peanut. Then there was the moderate severity patient who was suspected of being in an anergic state. The basophil upregulation in the mild and unexposed symptom group patients ranged from 0-5.8% which was well below the 10% cut-off. Peanut challenges to confirm allergy status could be beneficial in these patients as long as the blood work did not show significantly high levels of SIgE to components that were not testing in the BAT, ie, Ara h 3, Ara h 6, and Ara h 9. It is important to note, however, that those components should still be present in the whole peanut extract so to a certain degree so the BAT should still be somewhat predictive in those cases.

The number of discrepant results was also looked at to ensure that the 10% cut-off functioned well and to see if there were significant problems with the BAT tests. There were 22 cases where there was SIgE sensitization but no BAT upregulation and one case with BAT upregulation and no SIgE sensitization which was discussed earlier. These cases are summarized in Table 5.5. Two of the patients were our peanut challenge patients that had been proven to be tolerant to peanut and the discordant results were expected. Then there were 9 discrepant cases with whole peanut sensitization that ranged from 0.22 to 8.42 kU/l, 6 cases with Ara h 1 sensitization that ranged from 0.12 to 3.84 kU/l, 5 cases of Ara h 2 sensitization that ranged from 0.15 to 0.87 kU/l, and 5 cases of Ara h 8 sensitization that ranged from 0.28-2.36 kU/l.

One patient, PBAT 021, has the highest values of SIgE sensitization discrepancies for peanut, Ara h 1, and Ara h 2 (8.42, 3.84, 0.87 kU/l, respectively). The corresponding BAT values are 5.8%, 2.3%, and 2.8% which are much higher than we would expect to see in patients with no allergies since the average BAT upregulation on

Table 5.5 Discrepancies between SIgE Sensitization and BAT Results

| | SigE Sensitization | SigE Level (kU/l) | BAT% | Clinical Symptoms |
|---|---|---|---|---|
| PBAT 017 | Ara h 8 | 0.28 | 0.7% | Anaphylaxis |
| PBAT 018 | Ara h 1 | 0.00 | 68.7% | Anaphylaxis |
| PBAT 019 | Ara h 2 | 0.15 | 0.8% | EOE |
| PBAT 021 | Peanut | 8.42 | 5.8% | Not Exposed |
| | Ara h 1 | 3.84 | 2.3% | |
| | Ara h 2 | 0.87 | 2.8% | |
| PBAT 024 | Peanut | 0.80 | 0.9% | Not exposed |
| | Ara h 1 | 0.12 | 0.5% | |
| | Ara h 8 | 2.36 | 0.5% | |
| PBAT 025 | Ara h 8 | 1.10 | 0.0% | Respiratory Distress |
| PBAT 026 | Ara h 1 | 1.05 | 9.2% | Hives, Swelling |
| PBAT 028 | Peanut | 5.07 | 2.0% | Hives |
| | Ara h 1 | 0.12 | 2.0% | |
| PBAT 032 | Ara h 1 | 2.10 | 8.2% | Mouth tingling/itching |
| PBAT 033 | Peanut | 0.57 | 0.2% | Not exposed |
| PBAT 034 | Peanut | 0.45 | 9.5% | Hives, mouth swelling, itching |
| PBAT 037 | Peanut | 0.23 | 2.5% | Hives |
| PBAT 038 | Peanut | 1.48 | 0.0% | Oral Allergy Syndrome |
| PBAT 041 | Ara h 2 | 1.34 | 9.7% | Itching, Rash |
| PBAT 042 | Ara h 8 | 0.46 | 0.3% | Respiratory Distress |
| PBAT 044 | Peanut | 0.22 | 1.2% | Tolerant/Passed Challenge |
| PBAT 047 | Ara h 2 | 0.27 | 5.2% | Hives, Throat Tightening |
| PBAT 054 | Peanut | 0.63 | 0.3% | Hives, Respiratory issues |
| | Ara h 2 | 0.31 | 0.0% | |
| PBAT 055 | Ara h 8 | 0.41 | 0.0% | Tolerant/Passed Challenge |

the controls was 0.37%. PBAT 021 has never been exposed to peanuts as far as we know so it is hard to determine if a true allergy exists and whether or not the result should be classified as a false negative. A food challenge would also be beneficial in this case to correctly determine allergy status.

All of the Ara h 8 sensitized patients were negative in this study, as previously noted, and all but 1 patient was polysensitized with the other peanut components. The range of sensitization was also rather low and the highest sensitization was at 2.36 kU/l. In future studies, it might be beneficial to screen for those with higher levels (>15kU/l) to fully investigate whether Ara h 8 is capable of causing basophil activation in a polysensitized patient.

There were 4 patients whose BAT values were 9.2%, 8.2%, 9.5%, and 9.7% that were extremely close to the 10% cut-off. All 4 of the patients had convincing clinical history of symptoms to peanuts. The next closest upregulations to those 4 patients were at 5.2 and 5.8 % activation and only one of those lower responding patients had a clinical history to peanut. This, along with the mean BAT activation of <1% in the controls, indicates that the cut-off could be lowered to 8.0% and still retain good specificity for true peanut allergies. An "Undetermined—Interpret with Caution" range could also be created for values that fall in between the background cut-off level of 2.5% and the positive cut-off level until more data can be gather on these low-responding patients.

## Stability Studies

Stability studies to further evaluate the utility of BAT were performed during the study. Three patients, who were responsive to the controls, were selected at

random. The patient samples were tested within 9 hours of collection as per the research design. Then the samples were kept at room temperature and the three BAT control tests (background, anti- FcRI, and fMLP) were repeated at approximately 24 and 48 hours after the collection time.

The mean basophil recovery was 582, 600, and 574 at original BAT test time, and at 24 hours, and 48 hours, respectively. The recovery rate did not have a significant difference, $p = 0.92$ and $p = 0.93$ ($t$-test) between the different times and the background activation levels never went above 0.03 which was well below the 2.5% cut-off. The change in the percentage of basophils responding to the controls was evaluated. There was a mean overall decrease of 0.5% observed at 24 hours compared to the original BAT and a much greater mean decrease of 22% was noted at 48 hours. The calculated sample size for determining if a difference occurs between the two points was eighteen samples which was not achieved so statistical significance was not calculated. The lab values for the individual patients are shown in Table 5.6. There was some variability between the patients, but overall, they showed similar trends.

A responsiveness of at least 10% of basophils is needed to confirm an allergy. If we allowed patient testing at 48 hours, it would, therefore, need to have greater than 32% upregulation at the time of collection. Out of the 41 patients included in the final analysis, only 4 (9.8%) had an upregulation response of less than 32 % and greater than 10% (nonresponder cut-off) when originally tested. This suggests that it could be possible to test patient samples from outlying clinics and hospitals within 24-48 hours and achieve a clinically relevant result in the majority of cases. Larger stability studies looking at the effect of collection or transport conditions on BAT testing should still be performed.

Table 5.6 Stability of BAT Testing

| | PBAT #041 | PBAT #042 | NPBAT #005 | Average |
|---|---|---|---|---|
| *Within 9 hours after collection* | | | | |
| Basophil Recovery | 600, 600, 553 | 600,486, 600 | 600, 600, 600 | 582 |
| Background % | 0.3 | 0.3 | 0.0 | 0.2 |
| FcεRI CD63% | 61.5 | 92 | 63.3 | 72.3 |
| fMLP CD63% | 19 | 29.2 | 31.7 | 26.6 |
| *At ~24 hours after collection* | | | | |
| Basophil Recovery | 600, 600, 600 | 600, 600, 600 | 600, 600, 600 | 600 |
| Background % | 0.3 | 0.2 | 0.2 | 0.2 |
| FcεRI CD63% | 68.5 | 87 | 59.8 | 71.8 |
| fMLP CD63% | 38.5 | 64.3 | 52.5 | 51.8 |
| *At ~48 hours after collection* | | | | |
| Basophil Recovery | 600, 600, 600 | 600, 455, 515 | 600, 600, 600 | 574 |
| Background % | 0.2 | 0.2 | 0 | 0.2 |
| FcεRI CD63% | 25.8 | 81.3 | 43.8 | 50.3 |
| fMLP CD63% | 21.8 | 64.1 | 47.5 | 44.5 |

# CHAPTER 6

## SIGNIFICANCE OF FINDINGS

The goal of this study was to see if peanut allergy diagnosis, and by interference determination of severity, could be improved through the use of peanut component SIgE and BAT testing. This study showed that BAT testing can improve allergy diagnosis and would be a beneficial tool for physicians. In contrast to just using SIgE levels, the use of a basophil activation assay allows medical professionals to evaluate one of the main mechanisms of the allergic response rather than just evaluating the presence of SIgE. There were clear differences between atopic and true peanut allergy populations. The mean whole peanut BAT % was <1% for the negative controls and the overall value for the allergic population was 32.9%. When broken down by severity group, there were statistically significant differences in the peanut BAT means when compared to the atopic controls. There was also a significant difference between the mild and severe symptom groups ($p$=0.04). These are important findings and could help physicians predict how a child would react in a food challenge or prevent a food challenge from being done if the BAT suggests that they will have a strong allergic response that could be life-threatening. The research data also clearly show that patients with more severe allergies tended to reacted at lower concentrations of peanut in vitro and the literature has shown that

determination of these allergen threshold levels can be used for monitoring oral immunotherapies, sublingual immunotherapy, and anti-IgE therapies.

The BAT appears to have good specificity and sensitivity. We only had one instance of a BAT being positive when there was no SIgE antibodies (2.3%) and none of the atopic controls had positive BAT's despite having other allergies to tree nuts, shellfish, eggs, as well as atopic conditions like chronic hives, eczema, and mastocytosis. The major limitation of this study is that food challenges, the gold standard for determining allergy status, were not permitted so it is hard to determine true rates for false positive and false negative results, especially among those who have never been exposed to peanuts. Skin prick tests, however, are also quite sensitive in predicting a severe response, although not as well as oral food challenges.

The recruited sample population size of ~50 was based off of literature reviews of studies of a similar design to ours; however, the exclusion of the nonresponders and the need to sub-divide the patients into multiple groups left some groups with rather small sample sizes. Further research and patient recruitment of those that have elect to undergo a food challenge could be done to improve upon the results and make a better determination of whether the BAT is capable of predicting a true allergy and the severity of the symptoms that would be seen. These studies are currently being planned. Another study that would be beneficial and is currently being planned is to incorporate a wider selection of Ara h 8 sensitized patients to determine if Ara h 8 truly is a minor allergen in polysensitized patients like our research suggests.

REFERENCES


1. Saavedra-Delgado, A. The many faces of the peanut. *Allergy Proc*. 1989;10(4):291-294.

2. History of Peanuts & Peanut Butter. National Peanut Board Web Site. http://nationalpeanutboard.org/the-facts/history-of-peanuts-peanut-butter/ Updated June 11, 2015. Accessed December 13, 2015.

3. Koppelman SJ, Vlooswijk RA, Knippels LM et al. Quantification of major peanut allergens Ara h 1 and Ara h 2 in the peanut varieties runner, spanish, virginia, and valencia, bred in different parts of the world. *Allergy*. 2001;56(2):132-137.

4. Kang I, Gallo M, Tillman B. Distribution of allergen composition in peanut and wild progenitor species. *Crop Science*. 2007;47(3):997-1003.

5. Kang IH, Srivastava P, Ozias-Akins P, Gallo M. Temporal and spatial expression of the major allergens in developing and germinating peanut seed. *Plant Physiology*. 2007;144(2):836-845.

6. Maleki SJ, Chung SY, Champagne ET, Raufman JP. The effects of roasting on the allergenic properties of peanut proteins. *J Allergy Clin Immunol*. 2000;106(4):763-768.

7. Beyer K, Morrow E, Li XM et al. Effects of cooking methods on peanut allergenicity. *J Allergy Clin Immunol*. 2001;107(6):1077-1081.

8. Hefle SL, Helm RM, Burks AW, Bush RK. Comparison of commercial peanut skin test extracts. *J Allergy Clin Immunol*. 1995;95(4):837-842.

9. Shin DS, Compadre CM, Maleki SJ et al. Biochemical and structural analysis of the IgE binding sites on Ara h1, an abundant and highly allergenic peanut protein. *J Biol Chem*. 1998;273(22):13753-13759.

10. Pomes A, Butts C, Chapman M. Quantification of Ara h 1 in peanuts: why roasting makes a difference. *Clin Exp Allergy*. 2006;36(6):824-830.

11. Khan JI, Di R, Patel P, Nanda V. Evaluating disulfide crosslinking and pH-induced aggregation of Arachis hypogea 1 as components of peanut allergy. *J Agric Food Chem*.

2013;61(35):8430-8435.

12. Price DB, Ackland ML, Burks W, Knight MI, Suphioglu C. Peanut allergens alter intestinal barrier permeability and tight junction localisation in caco-2 cell cultures. *Cell Physiol Biochem*. 2014;33(6):1758-1777.

13. Nicolaou N, Murray C, Belgrave D, Poorafshar M, Simpson A, Custovic A. Quantification of specific IgE to whole peanut extract and peanut components in prediction of peanut allergy. *J Allergy Clin Immunol*. 2011;127(3):684-685.

14. Palmer GW, Dibbern Jr D A, Burks AW et al. Comparative potency of Ara h 1 and Ara h 2 in imunochemical and functional assays of allergenicity. *Clinical Immunol.* 2005;115(3):302-312.

15. Starkl P, Krishnamurthy D, Szalai K et al. Heating affects structure, enterocyte adsorption and signaling, as well as immunogenicity of the peanut allergen Ara h 2. *Open Allergy J*. 2011;9(4):24-34.

16. Maleki SJ, Viquez O, Jacks T et al. The major peanut allergen, Ara h 2 functions as a trypsin inhibitor, and roasting enhances this function. *J Allergy Clin Immunol*. 2003;112(1):190-195.

17. Bublin M, Kostadinova M, Radauer C, et al. IgE cross-reactivity between the major peanut allergen Ara h 2 and the nonhomologous Ara h 1 and Ara h 3. *J Allergy Clin Immunol.* 2013;132(1):118-124.

18. MoverareR, Ahlstedt S, Bengtsson U et al. Evaluation of IgE antibodies to recombinant peanut allergens in patients with reported reactions to peanuts. *Int Arch Allergy Immunol*. 2011;156(3):282-290.

19. Kleber-Janke T, Crameri R, AppenzellerU, Schlaak M, Becker WM. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int Arch Allergy Immunol*. 1999;119(4): 265-274.

20. Chen X, Wang Q, El-Mezayen R, Zhuang Y, Dreskin SC. Ara h 2 and Ara h 6 have similar allergenic activity and are substantially redundant. *Int Arch Allergy Immunol*. 2013;160(3):251-258.

21. Suhr M, Wicklein D, Lepp U, Becker WM. Isolation and characterization of natural Ara h 6: evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat. *Mol Nutr Food Res*. 2004;48(5):390-399.

22. Ballmer-Weber B, Lidholm J, Fernandez-Rivas M et al. IgE recognition patterns in peanut allergy are age dependent: perspectives of the EuroPrevall study. *Allergy*. 2015;70(4):391-407.

23. Ackerbauer D, Bublin M, Radauer C et al. Component-resolved IgE profiles in Austrian patients with a convincing history of peanut allergy. *Int Arch Allergy Immunol*. 2015;166(1):13-24.

24. Codreanu F, Collignon O, Roitel O et al. A novel immunoassay using recombinant allergens simplifies peanut allergy diagnosis. *Int Arch Allergy Immunol*. 2011;154(3):216-226.

25. Mittag D, Akkerdaas J, Ballmer-Weber BK et al. Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. *J Allergy Clin Immunol*. 2004;114(6):1410-1417.

26. Steinman H. ImmunoCAP allergen information: rBet v 1 PR-10, birch. ThermoScientific Website.http://www.phadia.com/en/Products/Allergy-testing-products/ImmunoCAP-Allergen-Information/Tree-Pollens/Allergen-Components/rBet-v-1-PR-10-Birch/. Acccessed January 29, 2016.

27. Asarnoj A, Nilsson C, Lidholm J et al. Peanut component Ara h 8 sensitization and tolerance to peanut. *J Allergy Clin Immunol*. 2012;130(2):468-472.

28. Lauer I, Dueringer N, Pokoj S et al. The non-specific lipid transfer protein, Ara h 9, is an important allergen in peanut. *Clin Exp Allergy*. 2009;39(9):1427-1437.

29. Krause S, Reese G, Randow S et al. Lipid transfer protein (Ara h 9) as a new peanut allergen relevant for a Mediterranean allergic population. *J Allergy Clin Immunol*. 2009;124(4):771-778.

30. Arkwright PD, Summers CW, Riley BJ, Alsediq N, Pumphrey RS. IgE sensitization to the nonspecific lipid-transfer protein Ara h 9 and peanut-associated bronchospasm. *BioMed Res Int*. 2013:746507

31. Arachis Hypogaea. WHO/IUIS allergen nomenclature subcommittee web site.http://www.allergen.org/search.php?Species=Arachis%20hypogaea. Updated July 7, 2015. Accessed January 29, 2016.

32. Platts-Mills TA. The allergy epidemics: 1870-2010. *J Allergy Clin Immunol*. 2015;136(1): 3-13.

33. Sicherer SH, Munoz-Furlong A, Sampson HA. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J Allergy Clin Immunol*. 2003;112(6):1203-1207.

34. Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol*, 2001;107(1):191-193.

35. Zhou Y, Wang, JS, Yang, XJ et al. Peanut allergy, allergen composition, and methods

of reducing allergenicity: A review. *Int J Food Sci*. 2013; http://dx.doi.org/10.1155/2013/909140. Accessed March 8, 2016.

36. Sicherer S, Burks A, Sampson H. Clinical features of acute allergic reactions to peanut and tree nuts in children. *Pediatrics*. 1998;102(1):e6.

37. Brough HA, Makinson K, Penagos M et al. Distribution of peanut protein in the home environment. *J Allergy Clin Immunol*. 2013;132(3):623-629.

38. Simonte SJ, Ma S, Mofidi S, Sicherer SH. Relevance of casual contact with peanut butter in children with peanut allergy. *J Allergy Clin Immunol*. 2003;112(1):180-182.

39. Sicherer SH, Munoz-Furlong A, Godbold J H, Sampson HA. US prevalence of self-reported peanut, tree nut, and sesame seed allergy: 11-year follow-up. *J Allergy Clin Immunol*. 2010;125(6):1322-1326.

40. Fox AT, Sasieni P, du Toit G, Syed H, Lack G. Household peanut consumption as a risk factor for the development of peanut allergy. *J Allergy Clin Immunol*. 2009;123(2):417-423.

41. Du Toit G, Roberts G, Sayre PH et al. Randomized trial of peanut consumption in infants at risk for peanut allergy. *N Engl J Med*. 2015;372(9):803-813.

42. Lack G, Fox D, Northstone K, Golding J. Factors associated with the development of peanut allergy in childhood. *N Engl J Med*. 2003;348(11):977-985.

43. Frazier AL, Camargo CA, Malspeis S, Willett WC, Young MC. Prospective study of peripregnancy consumption of peanuts or tree nuts by mothers and the risk of peanut or tree nut allergy in their offspring. *JAMA Pediatr*. 2014;168(2):156-162.

44. Ehlayel M, Bener A, Sabbah A. Is high prevalence of vitamin D deficiency evidence for asthma and allergy risks? *Eur Ann Allergy Clin Immunol*. 2011;43(3):81-88.

45. Akan A, Azkur D, Ginis T et al. Vitamin D level in children is correlated with severity of atopic dermatitis but only in patients with allergic sensitization. *Pediatr Dermatol.* 2013;30(3):359-363.

46. Miyake Y, Sasaki S, Tanaka K, Hirota Y. Diary food, calcium and vitamin D intake in pregnancy, and wheeze and eczema in infants . *Eur Respir J*. 2010;35(6):1228-1234.

47. Rudders SA, Espinola JA, Camargo Jr CA. North-south difference in US emergency department visits for acute allergic reactions. *Ann Allergy Asthma Immunol*. 2010;104(5):413-416.

48. NilssonL, Bjorksten B, Hattevig G et al. Season of birth as predictor of atopic manifestations. *Arch Dis Child*. 2007;76(4):341-344.

49. West CE. Gut microbiota and allergic disease: new findings. *Curr Opin Clin Nutr Metab Care*, 2014;17(3):261-266.

50. Gupta RS, Springston EE, Smith B, Warrier MR, Pongracic J, Holl JL. Geographic variability of childhood food allergy in the United States. *Clin Pediatr*. 2012;51(9):856-861.

51. Naleway AL. Asthma and atopy in rural children: is farming protective? *Clin Med Res*. 2004;2(1):5-12.

52. Hesselmar B, Aberg N, Aberg B, Eriksson B, Bjorksten B. Does early exposure to cat or dog protect against later allergy development? *Clin Exp Allergy*. 1999;29(5):611-617.

53. Salvi S. Pollution and allergic airways disease. *Curr Opin Allergy Clin Immunol.* 2001;1(1):35-41.

54. Diaz-Sanchez D, Proietti L, Polosa R. Diesel fumes and the rising prevalence of atopy: an urban legend? *Curr Allergy Asthma Rep*. 2003;3(2):146-152.

55. Johansson S, Nopp A, van Hage M et al. Passive IgE-sensitization by blood transfusion. *Allergy.* 2005;60(9):1192-1199.

56. Phan TG, Strasser SI, Koorey D et al. Passive transfer of nut allergy after liver transplantation. *Arch Intern Med*. 2003;163(2):237-239.

57. Legendre C, Calliat-Zucman S, Samuel D, Morelon S, Bismuth H, Bach JF, Kreis H. Transfer of symptomatic peanut allergy to the recipient of a combined liver-and-kidney transplant. *N Engl J Med*. 1997;337(12):822-824.

58. Needham J, Nicholas S, Davis C. Food allergies developing after solid organ transplant. *Pediatr Transplantation*. 2015;19(8):827-835.

59. Gern JE, Yang E, Evrard HM, Sampson HA. Allergic reactions to milk-contaminated "nondairy" products. *N Engl J Med*. 1991;324(14):976-979.

60. Food allergen labeling and consumer protection act of 2004 questions and answers. U.S. Food and Drug Administration website. http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106890.htm Updated July 18, 2006. Accessed January 15, 2016.

61. Hefle S.L, FurlongTJ, Niemann L, Lemon-Mule H, Sicherere S, Taylor SL.

Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. *J Allergy Immunol*. 2007;120(1):171-176.

62. Crotty MP, Taylor SL. Risks associated with foods having advisory milk labeling. *J Allergy Clin Immunol*. 2010;125(4):935-937.

63. Ballmer-Weber BK, Fernandez-Rivas M, Beyer K et al. How much is too much?: threshold dose distributions for 5 food allergens. *J Allergy Clin Immunol*. 2015;135(4):964-971.

64. Grimshaw K, King R, Nordlee J, Hefle S, Warner J, Hourihane J. Presentation of allergen in different food preparations affects the nature of the allergic reaction--a case series. *Clin Exp Allergy*. 2003;33(11):1581-1585.

65. Oppenheimer J, Durham S, Nelson H, Wolther OD.*Allergy diagnostic testing*. World Allergy Organization website. http://www.worldallergy.org/professional/allergic_diseases_center/allergy_diagnostic/Updated July 2014. Accessed February 2, 2016.

66. Tversky J, Chelladurai Y, McGready J, Hamilton R. Performance and pain tolerability of current diagnostic allergy skin prick test device. *J Allergy Clin Immunol Pract*. 2015;3(6):888-893.

67. Roberts G, Lack G. Diagnosing peanut allergy with skin prick and specific IgE testing. *J Allergy Clin Immunol*. 2005;115(6):1291-1296.

68. Cox L, Williams B, Sicherer S et al. Pearls and pitfalls of allergy diagnostic testing: report from the American College of Allergy, Asthma and Immunology/American Academy of Allergy, Asthma and Immunology specific IgE test task force. *Ann Allergy Asthma Immunol*. 2008;101(6):580-592.

69. Bodtger U, Jacobsen C, Poulsen L, Malling H. Long-term repeatability of the skin prick test is high when supported by history or allergen-sensitivity tests: a prospective clinical study. *Allergy*. 2003;58(11):1180-1886.

70. Pesonen M, Kallio MJ, Siimes MA, Ranki A. Allergen skin prick testing in early childhood: reproducibility and prediction of allergic symptoms into early adulthood. *J Pediatr*. 2015;166(2):401-406.

71. McCann WA, Ownby DR. The reproducibility of the allergy skin test scoring and interpretation by board-certified/board-eligible allergists. *Ann Allergy Asthma Immunol*. 2002;89(4):368-371.

72. Astier C, Morisset M, Roitel O et al. Predictive value of skin prick tests using recombinant allergens for diagnosis of peanut allergy. *J Allergy Clin Immunol*. 2006;118(1):250-256.

73. Peeters K, Koppelman S, van Hoffen E et al. Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? *Clin Exp Allergy*. 2007;37(1):108-115.

74. van Nieuwaal NH, Lasfar W, Meijer Y et al. Utility of peanut-specific IgE levels in predicting the outcome of double-blind, placebo-controlled food challenges. *J Allergy Clin Immunol*. 2010;125(6):1391-1392.

75. Bock S, Atkins F. The natural history of peanut allergy. *J Allergy Clin Immunol*. 1989; 83(5):900-904.

76. Skolnick HS, Conover-Walker MK, Koerner CB, Sampson HA, Burks W, Wood RA. The natural history of peanut allergy. *J Allergy Clin Immunol*. 2001;107(2):367-374.

77. Kim EH, Bird JA, Kulis M et al. Sublingual immunotherapy for peanut allergy: clinical and immunologic evidence of desensitization. *J Allergy Clin Immunol*. 2011;127(3):640-646.

78. Varshney P, Jones SM, Scurlock AM et al. A randomized controlled study of peanut oral immunotherapy: clinical desensitization and modulation of the allergic response. *J Allergy Clin Immunol*. 2011;127(3):654-660.

79. Vazquez-Ortiz M, Turner PJ. Improving the safety of oral immunotherapy for food allergy. *PediatrAllergy Immunol.* 2016;27(2):117-125.

80. Vickery BP, Scurlock AM, Kulis M et al. Sustained unresponsiveness to peanuts in subjects who have completed peanut oral immunotherapy. *J Allergy Clin Immunol*. 2014;133(2):468-475.

81. Burks AW, Wood RA, Jones SM et al. Sublingual immunotherapy for peanut allergy: long-term follow-up of a randomized multicenter trial. *J Allery Clin Immunol*. 2015;135(5):1240-1248.

82. Arpaia N, Campbel C, Fan X., Dikiy S, Veeken JV, deRoos P, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*. 2013;504(7480):451-455

83. Tang ML, Ponsonby AL, Orsini F et al. Administration of a probiotic with peanut oral immunotherapy: a randomized trial. *J Allergy Clin Immunol*. 2015;135(3):737-744.

84. Frohlich E, Mayerhofer R, Holzer P. Reevaluating the hype: four bacterial metabolites under scrutiny. *Eur J Microbiol Immunol.* 2015;5(1):1-13.

85. Boyle RJ, Robins-Browne RM, Tang ML. Probiotic use in clinical practice: what are the risks. *Am J Clin Nutr.* 2006;83(6):1256-1264.

86. Turcanu V, Stephens A, Chan S, Rance F, Lack G. IgE-mediated facilitated antigen presentation underlies higher immune responses in peanut allergy. *Allergy*. 2010;65(1):1274-1281.

87. Yalcin AD. An overview of the effects of anti-IgE therapies. *Med Sci Monit*. 2014;22(20): 1691-1699.

88. Chang T, Shiung Y. Anti-IgE as a mast cell-stabilizing therapeutic agent. *J Allergy Clin Immunol*. 2006;117(6):1203-1212.

89. Xolair medication guide. U.S. Food and Drug Administration website. http://www.fda.gov/downloads/drugs/drugsafety/ucm089829.pdf Accessed February 4, 2016

90. Nopp A, Johansson S, Ankerst J et al.Basophil allergen threshold sensitivity: a useful approach to anti-IgE treatment efficacy evaluation. *Allergy*. 2006;61(3):298-302.

91. Wu LC, Zarrin AA. The production and regulation of IgE by the immune system. *Nature Reviews*. 2014;14(4):247-259.

92. Amarasekera M. Immunoglobulin E in health and disease. *Asia Pacific Allergy*. 2011;1(1):12-15.

93. Dehlink E, Baker AH, Yen E, Nurko S, Fiebiger E. Relationships between levels of serum IgE, cell-bound IgE, and IgE-receptors on peripheral blood cells in a pediatric population. *PLoS One.* 2010;5(8):e12204.

94. Flinterman AE, Knol EF, Lencer DA et al. Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy. *J Allergy Clin Immunol*. 2008;121(3):737-743.

95. Vereda A, van Hage M, Ahlstedt S et al. Peanut allergy: clinical and immunologic difference among patients from 3 different geographic regions. *J Allergy Clin Immunol.* 2011;127(3):603-607.

96. Ackerbauer D, Bublin M, Radauer D et al. Component-resolved IgE profiles in Austrian patients with a convincing history of peanut allergy. *Int Arch Allergy Immunol*. 2015;166(1):13-24.

97. Ladics G, van Bilsen J, Brouwer H, Vogel L, Vieths S, Knippels L. Assessment of three human FcERI-transfected RBL cell-lines for identifying IgE induced degranulation utilizing peanut-allergic patient sera and peanut protein extract. *Regul Toxicol Pharmacol*. 2008;51(3):288-294.

98. Handlogten MW, Deak PE, Bilgicer B. Two-allergen model reveals complex relationship between IgE crosslinking and degranulation. *Chem Biol.*2014;21(11):1445-1451.

99. Hausmann O, Gentinetta T, Fux M, Pichler W, Dahinden C. (2011). Robust expression of CCR3 as a single basophil selection marker in flow cytometry. *Allergy*, 85-91.

100. Khanolkar A, BurdenSJ, Hansen B, Wilson AR, Philipps GJ, Hill HR. Evaluation of CCR3 as a basophil activation marker. *Am J Clin Pathol*. 2013;140(3):293-300.

101. Chirumbolo S, Vella A, Ortolani R et al. Differential response to human basophil activation markers: a multi-parameter flow cytometry approach. *Clin Mol Allergy*. 2008;6:12

102. Ebo D, Bridts C, Hagendornes M, Aerts N, De Clerck I, Stevens W. Basophil activation test by flow cytometry: present and future applications in allergology. *Cytometry B Clin Cytom.* 2008;74(4):201-210.

103. Ocmant A, Mulier S, Hanssens L et al. Basophil activation tests for the diagnosis of food allergy in children. *Clin Exp Allergy*. 2009;39(8):1234-1245.

104. Santos AF, Douiri A, Becares N et al Basophil activation test discriminates between allergy and tolerance in peanut-sensitized children. *J Allergy Clin Immunol*. 2014;134(3):645-652.

105. Nguyen KL, Gillis S, MacGlashan Jr. DW. A comparative study of releasing and nonreleasing human basophils: nonreleasing basophils lack an early component of the signal transduction pathway that follows IgE cross-linking. *J Allergy Clin Immunol*. 1990;85(6):1020-1029.

106. Yamaguchi M, Hirai K, Ohta K et al. Nonreleasing basophils convert to releasing basophils by culturing with IL-3. *J Allergy Clin Immunol*. 1996;97(6):1279-1287.

107. Knol EF, Mul FP, Kuijpers TW, Verhoeven AJ, Roos D. Intracellular events in anti-IgE nonreleasing human basophils. *J Allergy Clin Immunol*. 1992;90(1):92-103.

108. MacGlashan Jr D. Two regions of down-regulation in the IgE-mediated signaling

pathway in human basophils. *J Immunol*. 2003;170(10):4914-4925.

109. MacGlashen Jr D, Miura K. Loss of syk kinase during IgE-mediated stimulation of human basophils. *Allergy*. 2004;114(6):1317-1324.

110. Kepley CL, Youssef L, AndrewsRP, Wilson BS, Oliver JM. Syk deficiency in nonreleaser basophils. *J Allergy Clin Immunol*. 1999;104(2 Pt 1): 279-284.

111. Glaumann S, Nopp A, Johansson S, Rudengren M, Nilsson C. Basophil allergen threshold sensitivity, CD-sens, IgE-sensitization and DBPCFC in peanut-sensitized children. *Allergy*. 2012;67(2):242-247.

112. Glaumann S, Nilsson C, Johansson S et al. Evaluation of basophils allergen threshold sensitivity (CD-sens) to peanut and Ara h 8 in children IgE-sensitized to Ara h 8. *Clin Mol Allergy*. 2015;13(1):5.